INDUCIBLE LECTINS FROM HEMOLYMPH OF *Anticarsia gemmatalis* Hübner (LEPIDOPTERA: Noctuidae)

BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure page

ABBREVIATIONS USED IN TABLES

α linkage	α
acetyl	Ac
N-acetyl-D-galactosamine	GalNAc
N-acetyl-D-glucosamine	GlcNAc
N-acetyl-D-mannosamine	ManNAc
N-acetyl neuraminic acid (sialic acid)	NANA
asialofetuin	Afetuin
β linkage	β
bovine submaxillary mucin	BSM
2-deoxy glucose	2-deGlc
dextran sulfate	DSO_4
fetuin	Fet
formalin-treated	form
D-fucose	D-Fuc
L-fucose	Fuc
D-galactose	Gal
L-galactose	L-Gal
D-glucose	Glc
glutaraldehyde-treated	glut
human ABO erythrocytes	H (ABO)
human O erythrocytes	H(O)
α-lactose	α-Lac
β-lactose	β-Lac
lactose	Lac
D-mannose	Man
α-methyl galactose	α-MeGal
α-methyl mannoside	α-MeMan
ND	not determined
neuraminidase treated	asialo
oligosaccharide	oligo
porcine submaxillary mucin	PSM
rabbit erythrocytes	rab
raffinose	Raf
D-rhamnose	Rhm
L-rhamnose	L-Rhm
sucrose	Suc
trehalose	Tre
trypsin-treated	tryp

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The inducible hemagglutinins (lectins) of larval *Anticarsia gemmatalis* were studied. Although agglutinins were present in low levels in noninduced hemolymph, injection of hyphal bodies from *Nomuraea rileyi* and blastospores of other fungi induced appearance of high titers of these molecules in the hemolymph. The induced hemolymph was termed immune hemolymph. Injection of bacteria and viruses did not induce similarly high titers.

The lectin system appears to consist of a galactose-binding component and a fucose-binding component. Both components were isolated by a novel sequential affinity chromatography procedure. Immune hemolymph was diluted in buffer containing fucose and applied to a galactose-agarose

column. Galactose-binding molecules were eluted with buffer containing galactose. The fucose-binding proteins in the first eluent were dialyzed to remove fucose, applied to a fucose-agarose column and eluted with buffer containing fucose. The galactose agglutinin and a partially purified fucose agglutinin were obtained. When analyzed by SDS-PAGE, the agglutinins of two sugar specificities were found to have subunits of similar MW (around 45 kd). Native gel electrophoresis and gel permeation chromatography demonstrated that the native hemagglutinin had an MW >300 kd.

The hemagglutinin activity of the galactose lectin was inhibited by μmolar quantities of D-galactose and galactose-related sugars. The hemagglutinin activity of the fucose lectin required the presence of Ca^{++} and was inhibited by mM quantities of L-fucose. Neither lectin showed specificity for erythrocytes of the human ABO blood groups.

Opsonization of fungal blastospores possessing exposed galactose residues with immune hemolymph accelerated clearance of these particles from hemolymph. Opsonization of fungal hyphal bodies lacking exposed galactose residues with immune hemolymph, normal hemolymph did not effect clearance of these particles from hemolymph. Clearance may be a function of the galactose-binding hemagglutinin.

CHAPTER 1

LITERATURE REVIEW AND RESEARCH AIMS

This chapter reviews highlights of the current literature on lectins pertinent to this dissertation. It addresses the evolution that has occurred in the concept of a lectin over the last century and how these molecules are being studied in the 1990s. This review places special emphasis on animal lectins, insect lectins and fucose lectins. Known and surmised functions of lectins are discussed. The research presented in this dissertation is introduced and objectives of the study are presented.

Lectins

Lectin research began after Stillmark (1888) discovered hemagglutinating activity in extracts from castor beans. This proteinaceous carbohydrate-binding molecule was ricin. Since the initial discovery, lectins have been found in virtually every group of organisms investigated for their occurrence. The term lectin (from the Latin "lectus," to select) was coined by Boyd and Sharpleigh in 1954 to emphasize their selective binding to complex glycoconjugates. Because lectins are frequently detected by

their ability to agglutinate erythrocytes, until recently they were narrowly defined based on specificity for particular sugar moieties (frequently monosaccharides) and their bi- or polyvalency which permitted cross-linking of sugar residues. As cited in reviews by Gallagher (1984) and Pusztai (1987), Makaela (1957) recognized four groups of lectins based on configuration of the hydroxyl groups at the C-3 and C-4 position on the monosaccharide. This scheme ignored anomeric specificity at C-1, a property exhibited by lectins with specificity for the human ABO blood groups. Goldstein and Hayes (1978) attempted to improve this scheme by listing sugar binding in order of specificity and denoting any anomeric preference e.g. α-lactose > α-galactose. Goldstein et al., 1980, proposed a definition which required a lectin to have at least two sugar-binding sites. Gallagher (1984) presented a classification scheme based on whether 1) the lectin was inhibited by a monosaccharide (Class I or simpler binding mode lectin) or oligosaccharide (Class II or complex binding mode lectin); 2) the monosaccharide-inhibitable lectin showed requirement for end-chain monosaccharides (obligate exolectin) or recognized both end-chain and internal monosaccharides (facultative exolectin); 3) the lectins which recognized only specific sugar sequences (oligosaccharides) required homotypic sugar sequences or heterotypic sugar sequences. For the Class I lectins, the definition included a

requirement that the lectin binding be inhibited by greater than 50% in the presence of 10 mM or less of the inhibiting monosaccharide. In a review of plant lectins, Etzler (1985) expanded the definition to include monovalent carbohydrate binding proteins.

In 1988, Barondes proposed to redefine lectins simply as "carbohydrate binding proteins other than enzymes and antibodies" (Barondes, 1988, p. 482) because of the growing body of data on endogenous animal lectins which did not fit the classical definition. This new definition encourages investigators to look at lectins from the functional viewpoint that lectins are recognition molecules and Yoshizaki suggested additionally that the definition would prove useful in studying the "molecular evolution of carbohydrate-binding proteins" (Yoshizaki, 1990, p. 589). As pointed out by Sharon and Lis "four different monosaccharides can form 35,560 distinct tetrasaccharides, whereas four different amino acids or nucleotides can form only 24 tetrameric structures" (Sharon and Lis, 1989, p. 227). It has been hypothesized that "the specificity of many natural polymers is written in terms of sugar residues and not of amino acids or nucleotides" (Sharon, 1975, p. 26). Plant and animal lectins have been shown to possess exquisite specificity in their ability to distinguish subtle differences in carbohydrate structures and this suggests that it is a lack of knowledge of carbohydrate chemistry

that is limiting our ability to understand the true oligosaccharide specificity. Deciphering the information carried by oligosaccharides may represent a new frontier in biology. Feizi (1988) outlined strategies for decoding this information using oligosaccharides as antigens, receptors and probes.

Lectins are frequently named by several schemes. In one common method, the first letters of the genus and species are used and L (for lectin) or A (for agglutinin) is added. Component lectins are designated by letters or numbers. For example, the lectin from castor bean, Ricinus communis, is termed RCA. It consists of two types of molecules. The toxic A chain can be designated RCA-A and the agglutinin, the B chain, RCA-B. The components could also be termed RCL-I and RCL-II, RCL-A and RCL-B, or RCA-I and RCA-II. The common name of a lectin is usually derived from the species name and the suffix "-in" added e.g. ricin. Frequently, investigators working on a particular lectin have adopted their own conventional terminology which may not be consistent with that adopted for another lectin.

Purified and partially purified lectin preparations are widely available commercially. Two common sources are Sigma Chemical Co. (St. Louis, MO) and EY Laboratories (San Mateo, CA). A general source of information on lectins is the Sigma catalog (Sigma Chemical Co., St. Louis, MO). Perusal of the lectin section reveals most commercially available

lectins are of plant origin and reflects the relative abundance of investigations on plant lectins compared to those from other organisms. Several lectins from animal, bacterial and fungal sources are also commercially available. Also obvious are the broad sugar specificities exhibited by lectins. Additionally, lectins have been conjugated to agents like fluorescein isothiocyanate (FITC), biotin, peroxidase and colloidal gold for use in assay methods other than hemagglutination assays. Use of labelled lectins has allowed characterization of exposed surface carbohydrates of cells and microorganisms. Work conducted in this laboratory using such probes has been useful in delineating the surface carbohydrates of entomopathogenic fungi and insect cells (Pendland and Boucias, 1984, 1986b, in press).

Some of the well-studied plant lectins are interesting because their biochemical diversity provides prototypes for comparing and categorizing lectins from other species. Most lectins are composed of multiple subunits and are often glycosylated. The propensity of lectins to bind carbohydrate moieties, including those possessed by component subunits, makes it difficult to determine native molecular weight. One of the classic lectins, concanavalin A (Con A), is a product of jackbean (Canavalia ensiformis) seeds. The lectin is glycosylated and consists of 4 subunits of MW 26,000 but undergoes varying degrees of

aggregation depending on pH (Kalb and Lustig, 1968). Con A recognizes D-glucose and D-mannose residues of the α- anomer and requires the divalent cations Ca^{++} and Mn^{++} (Reeke et al., 1974). Although not useful in blood typing, Con A represents one of the few lectins which possesses mannose specificity (its recognition capabilities for mannose are 10 times greater than for glucose). Other interesting lectins are found in seeds of Bandeiraea simplicifolia. One component lectin consists of 5 possible combinations (isolectins) of subunits BS-I (A) and BS-I (B) and has an aggregate MW of 114 kd. The BS-I (A) subunit recognizes α-D-N-acetyl-galactosamine while the BS-I (B) subunit recognizes α-D galactose. The possible compositions of isolectins are A4:A3B1:A2B2:A1B3:B4 (Hayes and Goldstein, 1974; Murphy and Goldstein, 1977). The B. simplicifolia lectin system also has another component, BS-II, which recognizes α-D-N-acetyl glucosamine (GlcNAc) (Shankar Iyer et al., 1976). Wheat germ agglutinin (WGA) consists of two subunits of 36 kd and exhibits specificity for N-acetyl-glucosamine dimers and N-acetyl-neuraminic acid (Allen et al., 1973; Nagata and Burger, 1974). An L-fucose-binding lectin is produced by the asparagus pea, Lotus tetragonobolus. It consists of 10 subunits all of which exhibit affinity for α-L-fucose. They exist in the ratio of 4:2:4 and are of approximately the same MW (Yariv et al., 1967; Kalb, 1968). Another lectin system occurs in gorse,

Ulex europeus and is composed of two types of molecules: UEA-I has specificity for α-L-fucose while UEA-II in inhibited by N,N' acetylchitobiose and salicin (Matsumoto and Osawa, 1969, 1970; Osawa and Matsumoto, 1972).

Plant lectins are ubiquitous and seeds of the families of Leguminosae and Graminaceae are particularly rich sources. The biological functions of plant lectins have been reviewed by Pusztai (1987) but in general, the functions of these molecules are obscure although they may represent a significant percentage of the total protein of a given source. Recently two papers have appeared in the literature which may open up an entirely new area for the study of plant and insect herbivore relationships. Murdoch et al. (1990) studied the effect of plant lectins on the cowpea weevil and found that five of the 17 lectins screened delayed the development of these insects. Pratt et al. (1990) found that addition to artificial diet of purified lectin from the tepary bean (Phaseolus acutifolius) delayed development in the bruchid beetle, Acanthoscelides obtectus. Since most Phaseolus spp. are known to contain lectins, these proteinaceous chemicals can now be argued to play a role in plant defense against insect predation as suggested by Janzen et al. (1976).

Lectins can also be useful as tools (Sharon, 1987), and only a few actual applications and potential applications will be discussed in this review. Lectins are routinely

used as diagnostic reagents for typing of the human ABO blood system. Others, such as pokeweed mitogen and phytohemagglutinin, function as mitogens for subsets of lymphocytes and as such have been successfully used to stimulate cell division and antibody production in normally unreceptive B cells and for stimulating nonreceptive T cells. These properties have proven extremely useful for immunologists. The castor bean lectin, ricin, is being researched for use in immunotherapy as an immunotoxin (see Vitetta and Uhr, 1985, and Olsnes et al., 1989, for review). Ricin is known to consist of a toxin component which can recognize N-acetyl-D-galactosamine and β-D-galactose and an agglutinin component which recognizes β-D-galactose. In theory, an antibody is raised against a tumor specific antigen and this antibody is complexed to the ricin toxin. After this complex is administered to a patient, the antibody targets the tumor cells bearing the foreign antigen. After binding, the antibody-toxin complex is endocytosed by the cell which succumbs to the toxin.

In the animal kingdom, there has been an expanding body of data on endogenous lectins but many of these molecules failed to fit the classical definition of a lectin because they lacked cross-linking capability (valency). As mentioned above, Barondes (1988) proposed to redefine "lectin" to include these molecules. As animal lectins have become better studied, functional differences appeared and

two classes--membrane lectins and soluble lectins--could be distinguished (Barondes, 1984; Leffler et al., 1989; Caron et al., 1990). Membrane lectins require detergent solubilization prior to purification and apparently function to bind glycoconjugates to membranes. The soluble lectins can move freely and interact with both soluble and membrane-bound glycoconjugates. Recently, there has been growing interest in a family of soluble galactoside-binding vertebrate lectins. These can be subdivided into two classes: S-type lectins and C-type lectins (Drickamer, 1988). C-type lectins require Ca^{++}, possess cysteine molecules as disulfides, show variable solubility, are extracellular and have varying carbohydrate specificities. In contrast, the S-type lectins do not require Ca^{++} and possess cysteine molecules as free thiols. They are soluble in buffer and may exist intracellularly or extracellularly and have preference for terminal β-galactosides. These lectins will be discussed because they probably represent the prototypes of the lectins occurring in the Anticarsia gemmatalis system and in other insects.

As mentioned above, the C-type endogenous animal lectins have a requirement for Ca^{++}. Membership in this large family has been corroborated through amino acid sequence data. Vertebrate members include the asialoglycoprotein receptor, chicken hepatic lectin, mannose binding protein, and lymphocyte Fc receptor. Two

invertebrate lectins, the Sarcophaga peregrina lectin and sea urchin lectin, have also been included in this group. The common feature of such diverse molecules is a carbohydrate recognition domain (CRD) with 18 conserved amino acids within the 130 amino acid domain.

The S-type lectins are the second family of soluble animal lectins. They show no requirement for divalent cations but are usually assayed in the presence of thiols since oxidation inhibits carbohydrate binding activity. Another common feature of this group is molecule weight of 14-16 kd. Structural analysis of the carbohydrate domain reveals similarity within the group and no similarity to CRD of C-type lectins. Additionally, there are no cysteine residues in the invariant region.

Some of the endogenous animal lectins have a more complex nature than carbohydrate specificity. These have been reviewed by Barondes (1988). At least two, discoidin I, a lectin from the cellular slime mold Dictyostelium discoideum, and the elastin receptor can mediate both carbohydrate-protein and protein-protein interactions. Discoidin I is a classical lectin that is developmentally regulated. In the aggregating stage of this slime mold, the lectin is exocytosed in multilaminar bodies which form the matrix upon which the cells aggregate. The lectin also has a protein binding site which recognizes the tripeptide Arg-Gly-Asp and is essential for aggregate morphogenesis in this

organism. This tripeptide is identical to that required for substratum adhesion in fibronectin and laminin. The elastin receptor also shows bifunctionality since it can be affinity purified on either an elastin or glycoconjugate affinity matrix and eluted from these columns respectively by the peptide Val-Gly-Val-Ala-Pro-Gly or lactose (Wrenn et al. 1988; Hinek et al., 1988). These investigators have also suggested that the function of this receptor is to provide for the proper alignment of the elastin microfibrils to themselves and the extracellular matrix. One insect lectin has been reported to be bifunctional. The inducible lectin from Manduca sexta can function as a hemocyte coagulant (Minnick et al., 1986).

Lectins which recognize L-fucose are unique in that they recognize a biologically occurring sugar of the L enantiomer. A fucose lectin appears to exist in A. gemmatalis. For comparison with the A. gemmatalis fucose binding proteins, properties of known fucose lectins are summarized in Table 1-1. Although widely distributed in nature, fucose lectins are considered uncommon (Gilboa-Garber et al., 1988).

Insect Lectins

Hemagglutinins were first reported to occur in insects by Bernheimer (1952). He detected this activity in larvae and pupae in 10 of the 46 lepidopteran species examined.

Table 1-1. Properties of fucose lectins.

Lectin source (common name)(ref.)	MW in kd (native/subunit)	Blood Group Specific	Other Properties
Lotus tetragonobolus seeds(winged pea) (1)	120:58:117/ 33:29:29.25	yes	requires Ca^{++} ten subunits
Ulex europeus seeds (gorse) (2)	170/none	yes	requires Ca^{++}
Squilla mantis (crustacean) (3)	193/none	no	
Galactia tenuiflora seeds (?) (4)	72/27:29	yes	terminal fucose groups
Ulva lactica (green algae) (5)		yes H>>B>A>AB	L-Gal; L-Fuc; papain treated RBC
Aleuria aurantia (fungus) (6)	72	no	identical subunits
Streptomyces spp. (bacteria) (7)	60-70-180	H & B (except 1 strain)	MW depends on species
Metarhizium anisopliae (conidia) (8)	ND/32		
Anguilla anguilla (eel) (9)	40/20	yes	

References:

1) Kalb, 1968
2) Matsumoto and Osawa, 1969
3) Amirante and Basso, 1984
4) LePendu et al., 1986
5) Gilboa-Garber et al., 1988
6) Kochibe and Furukawa, 1980
7) Kameyama et al., 1982; Matsui et al., 1982
8) Boucias et al., 1988
9) Hořejší and Kocourek, 1978; Kelly, 1984;

None of the butterflies showed activity and the eggs and adults of the moths were similarly negative when tested against erythrocytes of various human blood groups (ABO, Rh,M and N). Invertebrate lectins continued to receive attention from numerous investigators (for reviews see Ratcliffe and Rowley, 1983; Rowley et al., 1986). By 1986, endogenous hemagglutinins had been found in six orders including 15 species and inducible agglutinins were reported from four species representing four orders (Ratcliffe and Rowley et al., 1986). Recently (1986), in Hemocytic and Humoral Immunity in Arthropods, edited by A.P. Gupta, Hapner and Stebbins summarized properties of purified lectins from seven insects in three orders. The chapter by Rowley et al., 1986, reviewed humoral recognition factors in insects. Since these reviews, lectins have been reported to occur in other insects. A summary of the well-studied insect lectins is presented in Table 1-2.

In most species, lectins have been detected in hemolymph but are known to occur in other tissues. These findings are summarized in Table 1-3. Most lectins have been found to occur constitutively but several inducible lectins are known. Known inducible insect lectins are listed in Table 1-4. In several insect species, agglutinins occur which recognize microorganisms. In Diptera, molecules have been found which agglutinate Trypanosoma, Leishmania, and Crithidia (Ingram et al., 1983; Ingram et al., 1984;

Ibrahim et al., 1984) and in the lepidopteran Philosamia ricini similar molecules are active against Bacillus thuringiensis (Bellah et al., 1988). In honeybees, agglutinins are synthesized in response to a bacterial pathogen (Bacillus larvae) (Gilliam and Jeter, 1970).

For the most part, the site of synthesis and function of insect lectins are poorly understood. Although it seems that insect hemagglutinins will exhibit the same broad range of specificities found in lectins from species in other kingdoms, a significant number of insect lectins appear to be specific for galactosyl residues.

The best studied insect lectin occurs constitutively in pupae of Sarcophaga peregrina (Komano et al., 1980, 1983) but can also be induced in larvae and adults (Kubo et al., 1984) by injury to the body wall. This activation has been shown to be mediated by a humoral factor in vivo and in cultured fat body (Shiraishi and Natori, 1988, 1989). It has been suggested that this galactose-inhibitable lectin functions during pupation in recognition of effete larval tissue and as a wound response protein in larvae (Komano et al., 1981). It has also assists in removal and lysis of foreign tissue (Komano and Natori, 1985). This lectin is synthesized by the fat body but can be detected on the surface of hemocytes. Studies have been expanded to cloning and sequencing of the lectin gene (Takahashi et al., 1985) and cloning and in vitro transcription of the lectin gene

Table 1-2. Hemagglutinin Activity in Insect Hemolymph.

Insect (life stage)(ref.)	MW in kd (native/subunit)	Ca++ reqmt.	Erythrocytes Agglutinated	Carbohydrate Inhibitors
Melanoplus sanguinipes (adult) (1)	500-700/ 70 (40:28)	yes	asialo human (ABO) rabbit; guinea pig calf, mouse, chick	D-Gal D-glucosides
Melanoplus differentialis (adult) (1)	(essentially the same as M. sanguinipes)			
Tellogryllus commodus (adult) (2)	>1,000/ 31:53	yes	human	NANA; GlcNAc GalNAc; fetuin
Extatasoma tiaratum (adult) (3)	ca 80		rabbit	Lac>Gal>α-MeGal> Raf> GalNAc
Schistocerca gregaria (adult) (4)			rabbit	Suc>Afetuin>L-Rhm
Periplaneta americana (adult) (5)			sheep	
Periplaneta americana (adult) (4)			human; rabbit	D-Fuc;Rhm>Lac;Glc

Table 1-2. Continued.

Insect (life stage)(Ref)	MW in kd (native/subunit)	Ca++ reqmt.	Erythrocytes Agglutinated	Carbohydrate Inhibitors
Periplaneta americana (adult) (6)		yes		α,β D-galactosyl; D-fucosyl;GalNAc; BSM; PSM; Afetuin
Locusta migratoria (larva-5) (7)		yes	rabbit > others	α-Gal pyranosides D-Fuc; α-1,6 oligo
Rhodnius prolixus (larva-5) (8)			rabbit	α,β GalNAc; Gal; GalNA; α-MeMan
Sarcophaga peregrina (pupa) (9)	190/32A:30B (2A:1B)2		sheep	Gal; Lac
Sarcophaga bullata (larva) (10)		no	human ABO, especially B	Gal; melibiose Gal derivatives
Glossina morsitans G.palpalis G.tachonoides (adult) (11)		no	human ABO	Sorbose; Tre; Glc 2-deGlc 1,4 link; 2-deGlc 1,6; α-link
Leptinotarsa decemlineata (larva,pupa) (12)		no	horse > others glut/tryps H(O) form H(O); rab; rat	sulfate containing; heparin; mucin; hexosamines; DSO_4

Table 1-2. Continued.

Insect (life stage) (ref.)	MW in kd (native/subunit)	Ca++ reqmt.	Erythrocytes Agglutinate	Carbohydrate Inhibitors
Leptinotarsa decemlineata (all stages) (13)	356/ 95.5:90	no	horse > others glut/trypsin H(O) form H(O); rab; rat	sulfate containing; heparin; mucin; hexosamine;dextranSO4
Allomyrina dichotoma I (larva) (14)	65/ 17.5:20	no	human	β-D-linked galactosides Gal; Lac lactulose
Allomyrina dichotoma II (larva) (14)	66.5/ 19:20	no	human	β-D-linked galactosides Gal; Lac lactulose
Bombyx mori (larva) (15)	260	no	sheep; tryp sheep glut sheep	glucuronic acid; heparin
Spodoptera exigua (larvae-5) (16)	ND/30.5;30	no	tryp rabbit	Gal; Lac
Anticarsia gemmatalis (larva) (17)		?	tryp rabbit human O	Gal; Lac; Fuc NANA
Hyalophora cecropia A (larva) (18)	160/40:41	?	rabbit; human; cow rat; asialo RBC	Gal; GalNAc (but not vs. rabbit)
Hyalophora cecropia B (larva) (18)	160/37:38	?	rabbit; human; cow	unknown

Table 1-2. Continued.

Insect (life stage)(ref.)	MW in kd (native/subunit)	Ca++ reqmt.	Erythrocytes Agglutinated	Carbohydrate Inhibitors
Anthereae pernyi (larva,pupa) (18)		?	rabbit; human; cow	Gal; GalNAc (but not vs. rabbit)
Anthereae pernyi (pupa) (19)	380/38	?	rabbit	Gal; GalNAc
Philosamia ricini (larva-5) (20)		?	human; guinea pig rat;	

References:

1) Stebbins and Hapner, 1985
2) Hapner and Jermyn, 1981
3) Richards et al., 1988
4) Lackie, 1981
5) Scott, 1972
6) Lackie and Vasta, 1988
7) Drif and Brehélin, 1989
8) Pereira et al., 1981
9) Komano et al., 1980
10) Stynen et al., 1985
11) Ingram and Molyneux, 1990
12) Stynen et al., 1982
13) Peferoen et al., 1982
14) Umetsu et al., 1982
15) Suzuki and Natori, 1983
16) Pendland and Boucias, 1986a
17) Pendland and Boucias, 1985
18) Castro et al., 1987
19) Qu et al., 1987
20) Bellah et al., 1988

Table 1-3. Hemagglutinin Activity in Insect Tissue other than Hemolymph.

Insect (life stage) (ref)	Tissue	Erythrocytes	Inhibitory Sugar
Pieris brassica (pharate adult) (1)	epidermal membrane; pharate wings	rabbit; tryp rabbit scorpion cuticle	GlcNAc; diNchitobiose; triN chitobiose
Galleria mellonella (larva) (2)	integument	rabbit; tryp rabbit	GalNAc (50 mM) Fuc (200 mM)
Rhodnius prolixus (larva) (3)	crop	rabbit	ManNAc
Rhodnius prolixus (larva) (3)	midgut	rabbit	Gal: GalNAc
Glossina morsitans G. palpalis G. tachonoides (adult) (4)	gut	human ABO	Glc; Gal Man derivatives
Periplaneta americana (adult male) (5)	muscle	human O	glycosaminoglycans
Drosophila melanogaster (adult; larva-3) (6)	whole insect homogenates	alcohol tr. glut fixed tryp rabbit	glucuronic acid heparin

Table 1-3. Continued.

Insect (life stage) (ref)	Tissue	Erythrocytes	Inhibiting Sugar
Calliphora erythrocephala (larva) (7)	peritrophic membrane (lumen side)		Man
Galleria mellonella (larva) (8,9)	hemocyte lysate (plasmatocytes)		β-1,3 glucan; laminarin, zymosan

References:

1) Mauchamp, 1982
2) Matha, 1989
3) Pereira et al., 1983
4) Ingram and Molyneux, 1990
5) Denburg, 1980
6) Ceri, 1984
7) Peters et al., 1983
8) Matha et al., 1990a
9) Matha et al., 1990b

Table 1-4. Inducible Hemagglutinins in Insects.

Insect (life stage) (ref)	Inducing Agent	Erythrocytes	Inhibiting Sugar
Sarcophaga peregrina (larva) (1)	injury to body wall	sheep	Gal; Lac
Anticarsia gemmatalis (larva) (2)	hyphal bodies of *Nomuraea rileyi*	tryp rabbit; tryp human O	Gal; Lac Fuc; NANA
Manduca sexta (larva) (3,4)	bacteria	sheep	Glc
Bombyx mori (larva) (5)	cytoplasmic polyhedrosis virus	tryp. glut. treated sheep	ND

References:

1) Natori *et al.*, 1980
2) Pendland and Boucias, 1985
3) Rupp and Spence, 1985
4) Minnick *et al.*, 1986
5) Mori *et al.*, 1989

(Kobayashi et al., 1989). Other elegant studies have demonstrated that a receptor for this lectin exists on the surface of mouse macrophages (Ohkuma et al., 1988) and that once stimulated, the macrophages are induced to produce a protein toxic to murine sarcoma cells (Itoh et al., 1986).

A constitutive galactose/glucose lectin exists in adult grasshoppers (Jurenka et al., 1982; Hapner, 1983; Stebbins and Hapner, 1985) and is essentially the same in both species studied (Melanoplus sanguinipes and M. differentialis). It is synthesized by the fat body (Stiles et al., 1988) and can be localized on the surface of hemocytes (Bradley et al., 1989). This lectin has been reported to lack an opsonic function but the surface carbohydrates of the test organism were not well-characterized and further studies need to be conducted.

In the cockroach Leucophaea maderae (Amirante and Mazzalai, 1978) and the giant silk moth Hyalophora cecropia (Yeaton, 1981), evidence for hemocytes as a synthetic source of lectin exists. The lectins of these insects have not been investigated for a function. The lectins from Hyalophora cecropia are of interest because superficially they appear similar to the group of isolectins found in the plant, B. simplicifolia. However, the A chain is specific for both galactose and N-acetylgalactosamine while the B chain recognizes an unknown moiety which may not be a carbohydrate. The MW is 160 kd with A subunits of 40 and 41

kd (N-acetyl-D-galactosamine and galactose) and B subunits of 37 and 38 kd (specificity unknown).

In this laboratory, Pendland and Boucias have purified a galactose lectin from Spodoptera exigua (Pendland and Boucias, 1986a) and demonstrated that it can function as an opsonin and enhance the phagocytosis of fungi which possess exposed galactose residues on their cell wall surfaces (Pendland et al., 1988). The site of synthesis of this constitutive lectin has yet to be explored. An inducible galactose lectin occurs in Anticarsia gemmatalis (Pendland and Boucias, 1985). Attempts to purify this lectin according to methods employed for the S. exigua lectin were unsuccessful and crossabsorption studies using human type O and rabbit erythrocytes demonstrated the relative complexity of this system. The purification and characterization of components of this lectin system is the subject of this dissertation and the research objective will be introduced at the end of this review.

Additionally, in this laboratory, various economically important noctuid larvae have been examined for the presence of hemagglutinins. In the Plusiinae, Pendland and Boucias (1985) failed to find lectin activity in hemolymph of larval Trichoplusia ni and Heath (unpublished) failed to find activity in Pseudoplusia includens. Challenge by intrahemocoelic injection of hyphal bodies from Nomuraea rileyi did not induce agglutinin production. In the

Agrotinae, both Heliothis zea and Heliothis virescens possess endogenous lectins with galactose specificity (Heath, unpublished). The naturally occurring hemagglutinin titers of H. virescens are higher (>16,000) than those of H. zea (range 16 - >1024) when assayed against trypsinized rabbit erythrocytes. The H. zea lectin is amenable to purification by affinity chromatography using a galactose-agarose resin and has essentially been purified. The MW is in the 30 kd range. The twice purified H. zea lectin is inhibited best by galactose and galactose derivatives but can also be inhibited by glucose and glucose related sugars. This activity is heat labile.

Since lectins and lectin-like molecules have been found on cell surfaces (either sequestered or synthesized by the cells), the lectin system found in the snail, Biomphalaria glabrata, is of interest in that it may serve as a prototype for the insect lectin function. Fryer et al. (1989) conducted experiments which can provide a model for opsonization and phagocytosis experiments. Using yeast (Saccharomyces cerevisiae) with known cell wall chemistry, these investigators presented evidence that the circulating hemolymph lectin and hemocyte recognition molecules exhibit different specificities. They postulated that the opsonic hemolymph factor (inhibitable by mannose) undergoes conformational changes once bound to yeast mannans and these changes subsequently allow binding to hemocyte surfaces

(inhibitable by β-1,3 glucan) and lead to enhanced phagocytosis (Bayne and Fryer, 1989). In molluscs the hemocytes are the putative site of lectin synthesis (Van der Knaap and Loker, 1990).

The recognition of β-1,3 glucans has been linked to nonself recognition in insects (Ashida et al., 1982) and this recognition system resides in the hemolymph and exists as a separate entity from the peptidoglycan recognition system in B. mori (Yoshida et al., 1986). Ochiai and Ashida (1988) have purified a β-1,3 glucan recognition protein from B. mori hemolymph. A β-1,3 glucan binding protein has also been isolated from Blaberus craniifer (Söderhäll et al., 1988). In the well-studied prophenoloxidase system of the crayfish, Duvic and Söderhäll (1990) purified a β-1,3 glucan binding protein from plasma. Recently, Maťha et al., 1990a, and Maťha et al., 1990b, reported the existence of a β-1,3 glucan lectin in Galleria mellonella. It was isolated from hemocyte lysate and localized in the plasmatocyte class of hemocytes. Immunocytochemical evidence suggested that it was synthesized in these cells also. As such, it may represent the nonspecific recognition factor found in insects. Additionally, Pendland et al. (1988) detected similar nonspecific binding of various microbial cells to plasmatocytes in S. exigua larvae.

Lectin Specificity and Function

As lectins have become better studied, more sophisticated methods are being used which argue for their exquisite specificity. Many immunochemical techniques have now been applied to the study of lectins and demonstrate that lectins show some analogous functions with immunoglobulins. These methods include use of equilibrium dialysis and Scatchard plots to determine valency and Michaelis-Menten experiments to determine binding kinetics. In insects, the Allomyrina dichotoma lectin is being studied by these methods (Yamashita et al., 1988). Recently, very elegant work has been performed by Bhattacharyya et al. 1989, 1990). These investigators found that purified lectin (Con A or LTA), in the presence of homogenous cross-linked glycopeptides, will precipitate and can be analyzed by quantitative precipitin analysis (Kabat and Mayer, 1961). Lectin, in the presence of heterologous complexes (two different glycopeptides), fails to precipitate and exist as soluble complexes. Inhibitory monosaccharides act as haptens and also exist as soluble complexes. These results can be analogous to the peptide-specific antibodies which, when purified, can be analyzed by quantitative precipitation techniques but exist physiologically as soluble complexes. If inhibited by haptens, the immunoglobulins also exist as soluble complexes. Further elegant work has been extended to view these complexes with the aid of the electron

microscope (EM). Each lectin subunit generally has one binding site and the valency of the molecule depends on the number of subunits. A biantennary oligosaccharide has two binding sites which may or may not be identical. These antennae may act analogously to the arms of the antibody Fab chains and have the ability to orient for proper cross-linking. With identical antennae, ordered arrays of a single type can be observed. With antennae of heterogenous composition, a different ordered pattern can be observed. These EM results corroborate data obtained from kinetic studies demonstrating that binding kinetics are specific for each glycopeptide inhibitor. It is also of interest that in vertebrates, many anti-carbohydrate antibodies exist constitutively. Frequently, the inhibitory carbohydrate haptens have been identified as blood group substances which an individual does not possess on his own tissue. These antibodies are generally of the IgM subclass and are unique in that they are not subject to class switching. Anti-carbohydrate antibodies of the IgG class can be prepared by injection of carbohydrates with MW above 10,000 daltons. Although there has been no demonstration of sequence homology between lectins and antibodies, work discussed above suggests the presence of domain homology within the large group of soluble vertebrate galactose lectins.

Since lectins show superficial functional similarity to antibodies, they have been frequently considered to function

as nonself recognition molecules in insects (Yeaton, 1981). Controversy continues over what can be considered an immune response in invertebrates (Klein, 1989; Marchalonis and Schluter, 1990). Insects are generally considered to lack immunoglobulins, but there have been reports (Harrelson and Goodman, 1988; Seeger et al., 1988; Bieber et al., 1989) of insect proteins which possess adequate sequence homology with immunoglobulins to place them in the immunoglobulin superfamily (Williams and Barclay, 1988); these proteins have been found to play roles in neural cell interaction. Sun et al., 1990, reported that hemolin (previously designated P4 by Boman, 1980) is an insect immune protein which belongs to the immunoglobulin superfamily also. It is induced by bacterial challenge in H. cecropia. Since the bacteria-induced P4 protein found in Manduca sexta shares the common characteristics of MW, isoelectric point, amino acid composition and N- terminal amino acid (Ladendorff and Kanost, 1990) with the H. cecropia P4 protein, perhaps such proteins are more widespread than previously thought.

Research Aims

Invertebrates are generally considered to lack specific adaptable humoral immunity, e.g. immunoglobulins and their cellular synthesizing machinery (lymphocytes), but are nonetheless known to possess an internal defense system which may include components for carbohydrate recognition

(lectins), nonself recognition, constitutive and inducible antibacterial proteins such as lysozymes, cecropins, attacins, hemolin, and β-1,3 glucan inducible components (prophenoloxidase system) (see Dunn, 1986 for review; Sun et al., 1990).

In 1985, Pendland and Boucias reported the existence of a galactose lectin in hemolymph of larval A. gemmatalis. The overall goal of the proposed research was to further understanding of the role lectins play in the insect humoral immune defense system by studying the novel inducible lectin of A. gemmatalis and exploring how it might function in the immune defense system of this insect. To this end, experiments were designed to effect maximum induction of the lectin and to subsequently develop an appropriate purification scheme for recovery of the lectin from the immune hemolymph. Once purified lectin was obtained, experiments would be set up to explore the sugar specificity of the lectin. Additionally, experimentation would be conducted to discover possible biological functions of the lectin in the insect or, if no function could be ascertained, then biological functions which could be ruled out would be determined. As with many systems, the A. gemmatalis lectin system could not be manipulated as easily as first anticipated and the results reported in this dissertation reflect where the system led the investigator and what was learned about the system during the course of

this research. As suspected by Pendland and Boucias (1985), the Anticarsia system was more complex than the system found in Spodoptera exigua. The fundamental difference between the anticipated and actual situation was demonstration of the existence of a second lectin or lectin-like molecule with specificity for L-fucose. The purification scheme which effected separation of these molecules yielded partially purified fucose lectin so both of these molecules were studied in tandem. For studies on biological function, whole immune hemolymph was used because it was felt that preliminary work should consider how the intact lectin system functions in the insect. Two general categories of biological function studies were designed: experiments to discover what agents caused induction of the lectin and experiments to determine whether the galactose-binding lectin could act as an opsonin.

CHAPTER 2

INDUCTION AND OCCURRENCE OF LECTIN IN *Anticarsia gemmatalis*

Introduction

The occurrence of a galactose/lactose hemagglutinin in *Sarcophaga peregrina* was reported by Natori *et al.* in 1980. This lectin occurred naturally in pupae but could be induced in larvae by injury to the body wall. In 1985, Pendland and Boucias published an account of an inducible galactose lectin in larval *Anticarsia gemmatalis*. Titers could be induced to higher levels by injection of hyphal bodies of the entomogenous hyphomycete *Nomuraea rileyi*. Subsequently, there have been only two additional reports of inducible insect lectins. In *Manduca sexta*, one of the antibacterial proteins, previously named M13 by Hurlbert *et al.* (1985) was found to have carbohydrate binding properties (Minnick *et al.*, 1986). This lectin has glucose specificity and bimodal activity, functioning also as a hemocyte coagulant. Its appearance in the hemolymph can be induced by hemocoelic injection of *Bacillus thuringiensis* (*B.t.*) or oral administration of a sublethal dose of the *B.t.* crystal toxin (Hurlbert *et al.*, 1985; Rupp and Spence, 1985). Mori *et al.* (1989) found a lectin in *Bombyx mori* which was induced by

cytoplasmic polyhedrosis virus administered *per os*. Since diverse agents have been implicated in lectin induction, experiments were designed to determine if agents other than *N. rileyi* were able to induce lectin production in *A. gemmatalis*.

Other studies were undertaken to look at constitutive lectin titers in 5th instar, 6th instar, wandering, prepupal and pupal *A. gemmatalis*. Natori *et al*. (1980) previously theorized that the *S. peregrina* lectin functioned in pupae in recognition of larval tissue. Bellah *et al*. (1989) reported that an age dependent lectin occurred in *Philosamia ricini*. This naturally occurring agglutinin was heat labile in early instars and heat stable in older larvae. It could also act as a bacterial agglutinin and this antibacterial activity was resistant to heating at 70 C in all larval stages studied suggesting a bimodal function for the molecule. The occurrence of a hemagglutinin, developmentally regulated by hemolymph ecdysteroids, has been recently reported to occur in *B. mori* (Amanai *et al*., 1990). The lectin exhibited specificity for glucuronic and galacturonic acid. This activity did not increase in response to injury to the body wall and reached maximum titers prior to larval-larval ecdysis and pupation. These investigators felt that the hemagglutinin played a role in post-embryonic development but not in immune defense.

Additional experiments were conducted to determine if lectin production could be induced by heat shock. Ezekowitz and Stahl (1988), in a review of vertebrate mannose lectin-like proteins, reported that these lectins were up-regulated in response to heat shock and could function as opsonins of mannose-bearing microorganisms. The mannose-binding proteins (MBPs) function in cooperation with a cell-bound mannose receptor which recognizes terminal mannose and fucose residues. Interestingly, vertebrate MBPs show some amino acid sequence homology with the S. peregrina lectin.

Materials and Methods

Insects maintenance. Colonies of A. gemmatalis were maintained in culture at the USDA Insectary in Gainesville, FL, and the insects were collected as eggs. They were reared on artificial diet (Greene et al., 1976) and housed in incubators at 26 C under a photoperiod of 14 hr light and 10 hr dark. For determination of instar, head capsule data kindly supplied by Dr. G. Wheeler was used.

Experimental design--induction of hemagglutinin. Insects were injected through a proleg with one of the various agents. Cohorts of 20 insects from the same treatment group were maintained with diet in paper cups until they were bled 24 or 48 hr post injection (PI). Ten individual insects were bled and an aliquot of 10 μl hemolymph diluted with 70 μl buffered insect saline (BIS)

(Castro et al., 1987). The samples were analyzed for hemagglutination activity. After bleeding, the cohorts of 20 insects were monitored for mortality.

Inducing agents. The fungi were chosen from cultures maintained in the Insect Pathology Laboratory. The Candida albicans strain, isolated from a human patient was provided by Dr. D. Soll (Iowa State University, Iowa City, IA). The Bacillus sphaericus strain 2362 was obtained from Mr. P. Vilarinhos (Brasilia, Brazil). The Escherichia coli (DH-5α) culture and Anticarsia gemmatalis nuclear polyhedrosis virus (AgNPV) was supplied by Ms. A. Garcia-Canedo. The AgNPV was from virus infected cell cultures and the amount of virus calculated by the following formula: 0.1 x $O.D._{260}$ (1 cm light path) x dilution = mg/ml (Dr. J. E. Maruniak, personal conversation).

Heat shock study. Groups of six insects were maintained at 30 or 37 C for 24 or 48 hr prior to bleeding.

Hemagglutination assay (HA). Hemagglutination was measured against trypsinized rabbit erythrocytes (RBC) and the titer expressed as the reciprocal of the highest dilution producing hemagglutination. A more detailed protocol is presented in Chapter 4 and in Appendix B.

Results

The responses of 6th instar larvae to challenge by various pathogenic and nonpathogenic microbes are shown in

Table 2-1. Hemolymph samples from individual insects were titered and a titer of 1024 was arbitrarily chosen as a high titer since only 25% of untreated insects had this amount of circulating hemagglutinin. Subsequent to injection of pathogenic and nonpathogenic fungi, high titers of lectin appeared in the hemolymph. In all treatments except one (P. farinosus 30,000 blastospores, 24 hr), the percentage of insects responding peaked and decreased by 48 hr PI. This treatment did show a slight decline from the 24 hr level when average titer was considered. The decrease in titer was most dramatic in 48 hr B. bassiana treated insects and although not shown, the HA profiles from these insects showed the reappearance of nonspecific ragged hemagglutination. This phenomenon was absent in all other insects challenged with fungi. The other agents--bacteria, virus, injury, saline--failed to induce similarly high titers of hemagglutinin. It is interesting that injection of the dipteran pathogen B. sphaericus produced only 35% mortality in A. gemmatalis.

Mortality data from these treatments are shown in Table 2-2. There was no attempt made to recover B. bassiana from treated insects but this fungus sporulates readily and can be isolated easily from cadavers. Results from one additional experiment are included in this table. Candida albicans (which is a vertebrate pathogen) was isolated from only one of the challenged larvae, and was reinjected into a

cohort of insects to determine if passage through a nonhost would enhance virulence for this nonhost. Passage did not enhance the virulence of this fungus.

The average titer was determined for three of the fungal agents and these results are shown in Table 2-3. Titers are the average titer of ten insects expressed as the $\log_2$ of the reciprocal of the dilution giving complete agglutination. The most effective agent for agglutinin induction was N. rileyi with a mean average titer of $2^{12.8}$ for 30,000 hyphal bodies at 24 hr.

Data on the occurrence of constitutive levels of hemagglutinin in various life stages are presented in Table 2-4. There is no evidence that the hemagglutinin is activated upon pupation. In fact, there is a decreased level of activity and the appearance of a prozone in hemolymph samples from these insects. Although sham injected insects showed an average titer of $2^{6.8}$ compared to saline injected controls with an average titer of 2^3, injury does not seem to induce lectin production.

Data from the heat shock experiment are shown in Table 2-5. The hemagglutinin does not seem to be a heat shock protein.

Table 2-1 Hemagglutinin activity in hemolymph of larval *Anticarsia gemmatalis* challenged with various microbial agents.

Treatment	Dose	Time of Sampling	% Response >8	>1024
N. rileyi	30,000	24 hr	90	80
	30,000	48 hr	100	70
	60,000	24 hr	100	60
	60,000	48 hr	100	30
	dusted	9 da	100	58
C. albicans	30,000	24 hr	100	70
	30,000	48 hr	100	40
	60,000	24 hr	100	80
	60,000	48 hr	100	20
B. bassiana	30,000	24 hr	100	40
	30,000	48 hr	100	20
	60,000	24 hr	100	80
	60,000	48 hr	80	10
P. farinosus	30,000	24 hr	100	30
	30,000	48 hr	80	70
	60,000	24 hr	100	80
	60,000	48 hr	100	50
E. coli	1×10^6	24 hr	80	0
	1×10^6	48 hr	100	0
B. sphaericus	1×10^6	24 hr	20	0
	1×10^6	48 hr	50	0
AgNPV	0.53 mg/ml	24 hr	80	20
		48 hr	80	20
sham		24 hr	70	30
		48 hr	30	10
saline		24 hr	20	10
		48 hr	30	10
untreated		24 hr	67	25

Table 2-2. Mortality of insects challenged with various microbial agents.

Treatment	Dose	%Mortality	Time of Death (da)	% Recovery of Agent
N. rileyi	30,000	100	3	100
	60,000	95	3	100
N. rileyi	dusted	100	9	100
C. albicans	30,000	53	3	*
	60,000	46	3	
C. albicans	30,000	30	3	0
(from insect)	60,000	30	3	0
B. bassiana	30,000	90	3	ND
	60,000	97.5	3	ND
P. farinosus	30,000	89	3	0
	60,000	91	3	0
E. coli	1×10^6	17.5	var.	0
B. sphaericus	1×10^6	35	var.	0
AgNPV		100	3-7	ND
sham		25	var.	
saline		10	var.	

* 1/73 insects (1.4%); ND - not determined; var. - variable

Table 2-3. Average titers of fungal challenged insects.

Treatment	Dose	Time	Average Titer*
N. rileyi	30,000	24 hr	12.6
	30,000	48 hr	11.8
	60,000	24 hr	11.3
	60,000	48 hr	9.9
B. bassiana	30,000	24 hr	8.9
	30,000	48 hr	8.5
	60,000	24 hr	12.0
	60,000	48 hr	3.9
P. farinosus	30,000	24 hr	9.7
	30,000	48 hr	8.8
	60,000	24 hr	11.9
	60,000	48 hr	10.3

* average of titers from 10 insects expressed in $\log_2$ of reciprocal of dilution giving complete hemagglutination

Table 2-4. Occurrence of hemagglutinin in life stages of Anticarsia gemmatalis.

Life stage	# Insects	% Response		Prozone
		>8	>1024	
Pupae	10	30	0	yes
Prepupae	10	100	30	no
Wandering	10	100	40	no
6th Instar	12	67	25	yes
5th Instar	12	100	83	yes

Table 2-5. Occurrence of hemagglutinin in larvae of A. gemmatalis after heat shock.

Temperature	# Insects	Length of Treatment	% Response	
			>8	>1024
30 C	6	24 hr	33	0
	6	48 hr	33	33
37 C	6	24 hr	33	0
	6	48 hr	100	0

Discussion

To date, there have been few reports of inducible lectins in insects (see Table 1-4). Induction by fungi has only been reported in larval A. gemmatalis (Pendland and Boucias, 1985). Unlike S. peregrina, the hemagglutinin does not seem to exist constitutively in pupae but average titers of $2^{10.4}$ and $2^{9.5}$ are found in wandering prepupae and prepupae, respectively. Thus, if production is developmentally regulated, the appearance of high lectin titers in hemolymph may occur at a different life stage than in the dipteran S. peregrina, and the A. gemmatalis lectin may be more closely related to the hemagglutinin found in B. mori than to the hemagglutinin found in S. peregrina. The function of the hemagglutinin in prepupae of A. gemmatalis is unknown. Since both pathogenic and nonpathogenic fungi can induce production of high titers of the lectin(s), the induction may be a response to fungal mannans and galactomannans which are recognized as nonself. The prophenoloxidase system in insects is generally considered to recognize another fungal wall component, β-1,3 glucan. Pendland and Boucias (1985) found that laminarin (β-1,3 glucan) did not induce lectin production. The prophenoloxidase system in A. gemmatalis is not as active as the system found in Trichoplusia ni (Boucias and Pendland, 1987), and challenge with N. rileyi hyphal bodies renders it even less active (personal observation). Once hemocytes are

removed from hemolymph, there is very little tendency for the hemolymph to melanize, while the surface layers of the pelleted hemocytes do eventually melanize. Furthermore, larval A. gemmatalis, unlike certain other insects, do not produce melanized nodules in response to injection of hyphal bodies or mycelia of N. rileyi (unpublished observation).

CHAPTER 3

LECTIN PURIFICATION AND PARTIAL CHARACTERIZATION

Introduction

The existence of a novel inducible galactose lectin in larval Anticarsia gemmatalis was reported by Pendland and Boucias (1985). Crossabsorption studies using trypsinized type O human erythrocytes and trypsinized rabbit erythrocytes provided evidence that the lectin was either multispecific (a functionally heterogeneous molecule) or a heteroagglutinin (structurally different agglutinin molecules). In 1986, these investigators reported the characterization of a galactose-specific hemagglutinin from larval Spodoptera exigua and were able to purify this lectin to homogeneity on an Affi-Gel ovalbumin column using an EDTA elution protocol. Initial attempts to purify the A. gemmatalis lectin by a similar method demonstrated the relative complexity of the A. gemmatalis lectin system. Many methods were attempted to effect purification and these are discussed in Appendix A. Although purification was not accomplished by these protocols, many of the methods provided data on the lectins. This chapter chronicles the

development of the protocol currently used in this laboratory for purification of the A. gemmatalis lectin(s).

Materials and Methods

Insect maintenance. Colonies of A. gemmatalis were maintained in culture at the USDA Insectary in Gainesville, FL. The insects were collected as eggs and reared on artificial diet (Greene et al., 1976) under a photoperiod of 14 hr light and 10 hr dark at 26 C.

Fungal culture maintenance. Strains of Nomuraea rileyi and other fungal cultures are stored at -70 C in the Insect Pathology Laboratory and are maintained on Sabouraud Maltose Yeast agar (SMY) or Sabouraud Dextrose Yeast broth (SDY). The strain used in this study was the FL-78 strain which was originally isolated from field collected A. gemmatalis larvae (Boucias et al., 1982)

Lectin induction. For injection, fungal cells were harvested as hyphal bodies (HB) by flooding the Petri plate with sterile water. Using aseptic technique, the fungal cells were washed several times in water and suspended in sterile 0.85% NaCl. After an additional centrifugation, the cells were resuspended in sterile saline with a vortex mixer. The cells were counted with a hemacytometer and diluted to the desired concentration. For preparation of high lectin titer serum, late sixth instar A. gemmatalis larvae were each inoculated with 30,000 washed HB in sterile

saline. Injections of 5-10 µl were made into a proleg with an ISCO injector (Instrumentation Specialties Co., Lincoln NE) equipped with a tuberculin syringe fitted to a 30 gauge needle (Thomas Scientific, Swedesboro, NJ). The insects were bled at 24 hr postinjection (PI) by puncturing a proleg. The hemolymph was collected on a sheet of parafilm placed on an ice bath. Hemolymph was pooled in a prechilled microcentrifuge tube containing a few crystals of phenylthiourea (PTU) and centrifuged at 10,000 x g for 5 min. The cell free hemolymph was pooled and stored at -70 C until processed. An average of 25 ul hemolymph could be obtained from each larva.

Lectin purification. A novel sequential affinity purification scheme was developed for purification of the A. gemmatalis lectin(s). Both a galactose-agarose resin (Pierce Chemical Co., Rockford, IL) and a fucose-agarose resin (Sigma Chemical Co, St. Louis, MO) were employed. Aliquots of 5 ml of pooled immune hemolymph were diluted 1:2 in buffered insect saline (BIS) (Castro et al., 1987) + 0.2 M L-fucose (fucose-BIS) and applied to a packed galactose-agarose resin which had been equilibrated with fucose-BIS. Approximately 15 ml of the eluent containing the fucose-binding proteins (FBPs) were collected and dialyzed extensively against BIS. The galactose-binding proteins (GBPs) remaining on the column were washed with BIS + 0.5 M NaCl (salt-BIS), fucose-BIS and the GBPs eluted with 0.4 M

galactose in BIS (galactose-BIS). Elution was monitored by increase in absorbance at 280 nm and hemagglutination (HA) assays. The GBPs were extensively dialyzed against BIS. The resin was washed with salt-BIS and BIS and equilibrated with fucose-BIS. The dialyzed GBPs were repurified by a second passage through the galactose-agarose column according to the protocol described above.

The dialyzed (FBPs) were applied to a packed fucose-agarose resin equilibrated with BIS. The protein-rich first eluent was reserved for other experimentation and the adhering FBPs were washed with salt-BIS, BIS and finally eluted with 0.4 M fucose in BIS. Elution was monitored by increase in absorbance at 280 nm and HA assays. The FBPs were extensively dialyzed against BIS and repurified according to the fucose agarose protocol. The resin was extensively washed with salt-BIS and BIS and equilibrated prior to each use.

The twice purified GBPs and FBPs were dialyzed against several changes of 1/10 BIS followed by dialysis against 1/50 BIS. The dialyzed fractions were then lyophilized and reconstituted with approximately 1 ml sterile deionized water.

Lectin purification using affinity chromatography followed by gel permeation chromatography. For the first step, a modification of the above affinity chromatography procedure was utilized. An aliquot of 2.5 ml of immune

hemolymph was diluted with an equal volume of galactose-BIS and applied to the fucose-agarose column using galactose-BIS as buffer. The FBPs were eluted with BIS+ 0.4M fucose. Two column runs (5 ml immune hemolymph) were pooled, dialyzed and lyophilized. The first eluents from the fucose-agarose column were pooled, dialyzed to remove the galactose and reapplied to a galactose-agarose column. The GBPs were eluted with BIS + 0.4 M galactose, dialyzed and lyophilized. Each lyophilized agglutinin was reconstituted with 300 μl of deionized water. A 200 μl sample was diluted with 200 μl BIS with 5% glycerol and applied to a 1 cm x 54 cm column of Sephacryl S-300 gel filtration media (Pharmacia Fine Chemicals, repackaged by Sigma, St. Louis, MO) in separate experiments. Fractions of 2 ml were collected and pooled based on HA activity and absorbance at 280 nm. For convenience, the fractions were designated from strip chart measurements as the distance, in cm, from the point of sample application to the points at which fraction collection was initiated and terminated. This distance measurement, although arbitrary, allowed for comparison of the fractions since the conditions for chromatography were kept uniform. The chart speed was 2 mm/min and the flow rate was 12 ml/hr. Molecular weight standards were also run under the same conditions as the samples but were found not to be particularly helpful in this study. Under these conditions, thyroglobulin (MW 669 kd; 330 kd for half unit)

eluted at 4.4 cm, apoferritin (MW 440 kd; 220 kd for half unit; 18.5 kd for subunit) eluted at 5.1 cm, β-amylase (MW 220 kd) eluted at 5.6 cm, alcohol dehydrogenase (MW 150 kd) eluted at 6.1 cm, bovine serum albumin (MW 66.2 kd) at 6.4 cm and carbonic anhydrase (MW 31 kd) eluted at 7.3 cm. The fractions were dialyzed, lyophilized and reconstituted. Samples of the fractions were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and stained with Coomassie Brilliant Blue. The remainder of each sample was tested for protein, hemagglutination and hemagglutination inhibition.

Hemagglutination assay (HA). Hemagglutination was measured against trypsinized rabbit erythrocytes (RBC) and the titer expressed as the reciprocal of the highest dilution producing hemagglutination. A more detailed protocol is presented in Chapter 4 and in Appendix B.

Hemagglutination Inhibition assay (HI). Hemagglutination inhibition was measured as the ability of the sample, serially diluted in either 0.2 M fucose-BIS or 0.2 M galactose-BIS to inhibit HA of the rabbit RBC. A more detailed protocol is presented in Chapter 4 and in Appendix B.

Sodium dodecyl Sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) and as outlined in the

Hoefer Protocols (Hoefer Scientific Instruments, San Francisco, CA). Both standard gels (Protean Apparatus, BioRad, Richmond, CA) and minigels (BioRad, Richmond, CA) were used. Molecular weight standards (BioRad) were run on each gel.

Native gel electrophoresis. A 4-20% gradient gel was prepared according to a method supplied by Dr. P. Greany. Molecular weight standards (Pharmacia Fine Chemicals, Uppsala, Sweden) were run on each gel.

Protein assays. The protein content of various samples was determined by the method of Bradford (1976) (Pierce Coomassie Assay) or the BCA method (Pierce BCA Assay) using a bovine serum albumin (BSA) standard. The reagents were purchased from Pierce Chem. Co., Rockford IL. During chromatography, protein was detected by absorbance at 280 nm.

Results

The protocol used for purification and partial purification of the lectin(s) from A. gemmatalis was developed after extensive experimentation as discussed in Appendix A. The initial successful experiment involved a single passage through the fucose agarose column in the presence of galactose and a single passage of the dialyzed first eluent through the galactose resin. These preparations were subjected to gel permeation on a Sephacryl

S-300 column. Figures 3-1 and 3-2 show the absorbance profile of the FBPs and GBPs eluting from the Sephacryl S-300 gel permeation column. Fractions were pooled based on HA titer and absorbance data. Figure 3-3 depicts the elution profile of the HA activity from each column. The purpose of this graph is to illustrate the relative position on the column where the activity eluted. As with the BioGel 1.5A column (Appendix A), molecular weight standards were not particularly useful for determining the MW of hemagglutinins because the HA activity did not elute in a sharp peak so that a native MW could be determined. For the fucose lectin, the peak activity eluted from 3.9 - 5.0 cm and 5.0 cm - 6.2 cm. At least some of the activity is in the MW range of thyroglobulin (669 kd) and the thyroglobulin half unit (330 kd). When the galactose lectin preparation was analyzed, the majority of the protein eluted 4.2 - 7 cm range but the majority of activity eluted from 8.5 - 9.7 cm which was beyond the point of elution of carbonic anhydrase (MW 31 kd). Because of the difficulty in representing serial twofold dilutions in such a format, high activity is not adequately represented on this graph.

Table 3-1 and Table 3-2 show the amount of this activity which could be inhibited by fucose or galactose, respectively. When the same fractions were electrophoresed on an SDS-PAGE gel, the profiles in Figures 3-4 and 3-5 were obtained. The results from these experiments demonstrated

that there existed a fraction in each preparation which could be presumed, by a process of elimination, to be the candidate lectin fraction (Fraction II of the FBPs and Fraction IV of the GBPs). These putative lectin fractions were of similar MW (about 45 kd) after SDS-PAGE. The fucose lectin appeared to consist of isolectins and the diffuse migration exhibited by the galactose lectin was suggestive of a glycoprotein. Native gels were run on the same preparations and the semipure fractions from SDS-PAGE showed a band in the 400 kd range. Because of inefficiency in purification and the inability to obtain discrete pure fractions by gel permeation, it was decided to pursue a double affinity purification according to the protocol described above.

Initially the protocol utilized a fucose-agarose purification followed by galactose-agarose purification because of the cost of including L-fucose in the buffer. When a galactose-agarose followed by fucose-agarose protocol was adopted, much cleaner preparations were obtained and the consistency in purification of the galactose lectin was greater.

To determine the efficiency of the purification protocol, three aliquots of 5 ml of immune hemolymph were used for the analysis. These results are shown in Table 3-3, Figure 3-6 and 3-7. The banding pattern of FBPs on the SDS-PAGE gels were not in agreement with previous work.

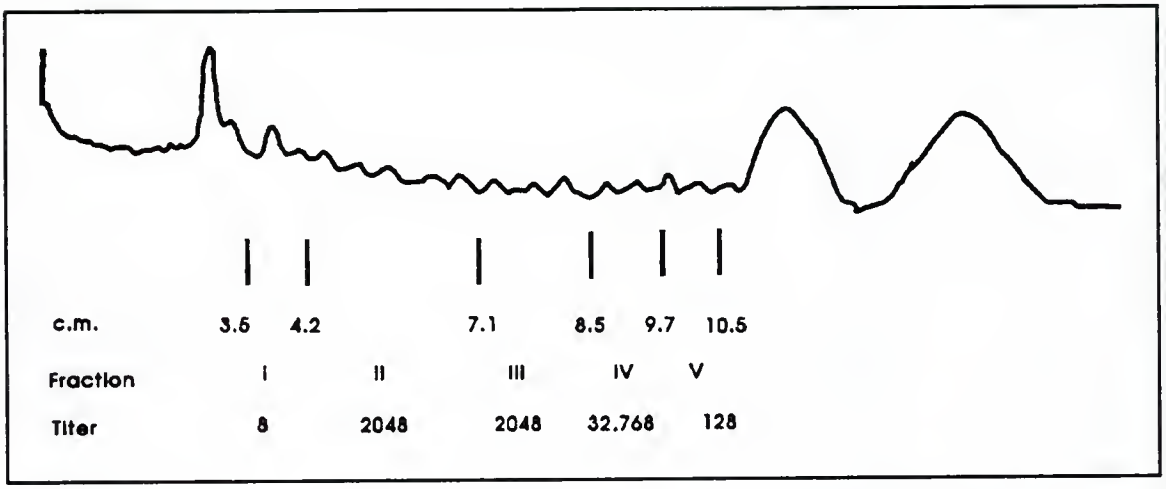


Figure 3-1. Absorbance profile (280 nm) of galactose binding proteins from Sephacryl S-300. Titer refers to hemagglutination titer of rabbit erythrocytes.

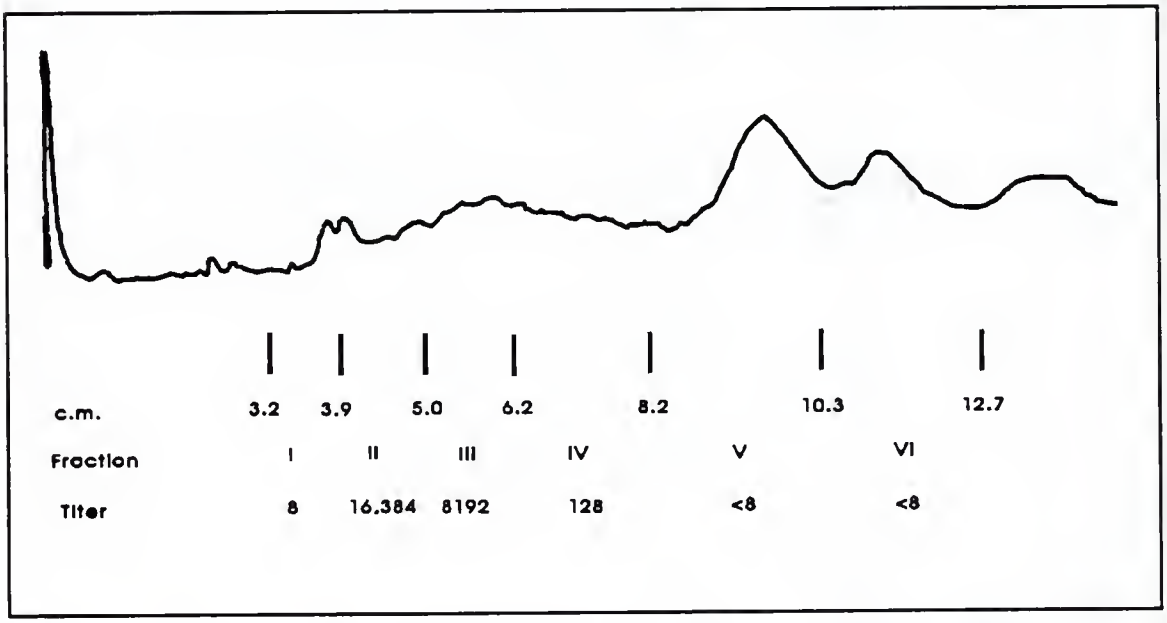


Figure 3-2. Absorbance profile (280 nm) of fucose binding proteins from Sephacryl S-300. Titer refers to hemagglutination titer of rabbit erythrocytes.

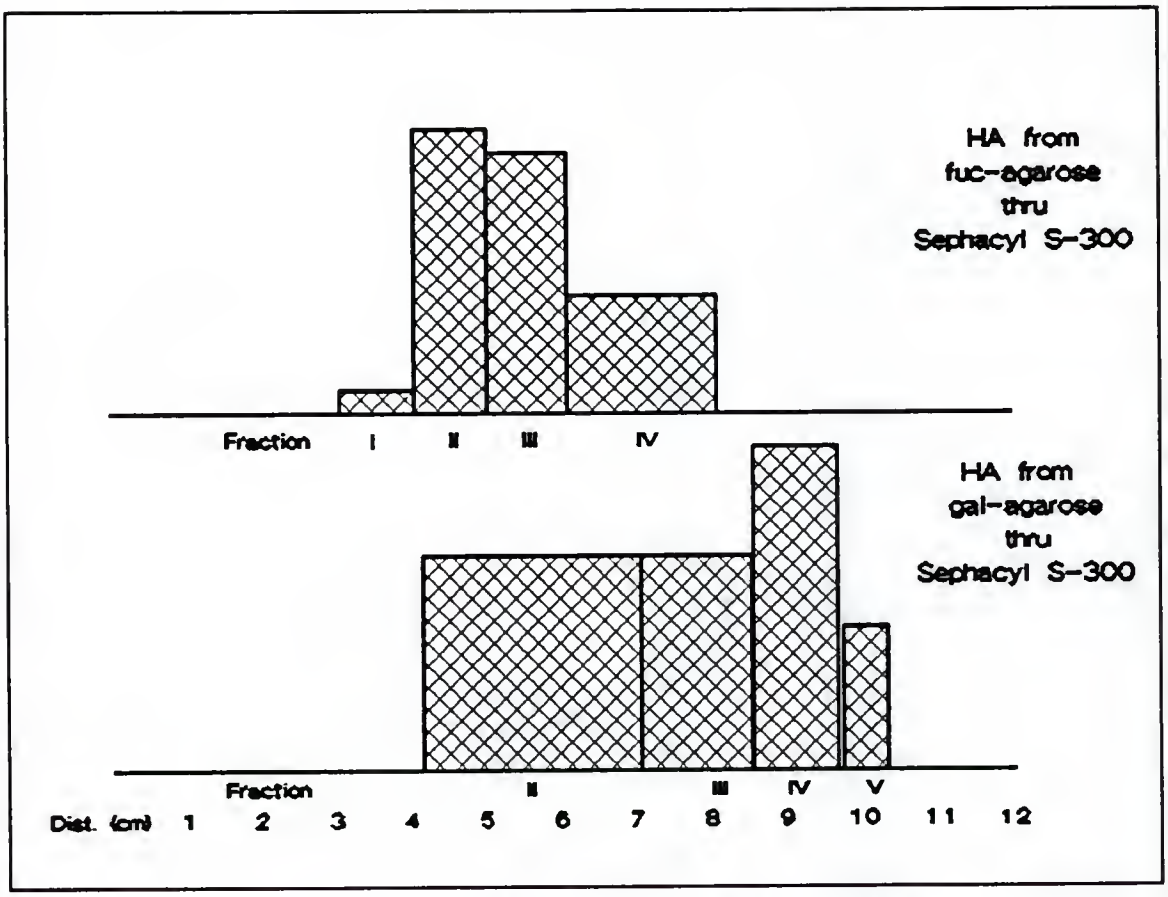


Figure 3-3. Hemagglutinin activity recovered from pooled fractions eluted from Sephacryl S-300.

Table 3-1. Inhibition by galactose and fucose of fractions of fucose binding proteins eluting from Sephacryl S-300. Total units (U) were calculated as titer x volume.

Fr.	ml	Titer	Total U HA activity	# U inh. by Gal	# U inh. by Fuc
I	6	8	48	0	48
II	12	16,384	196,608	98,304	196,608
III	12	8,192	98,304	0	98,304
IV	20	128	2,560	0	2,560
V	22	<8	0	0	0
VI	24	<8	0	0	0

Table 3-2. Inhibition by galactose and fucose of fractions of galactose binding proteins eluting from Sephacryl S-300. Total units (U) were calculated as titer x volume.

Fr.	ml	Titer	Total U HA activity	# U inh. by Gal	# U inh. by Fuc
I	6	<8	0	0	0
II	30	2048	61,440	61,440	960
III	14	2048	28,672	28,672	224
IV	12	32,768	393,216	393,216	768
V	8	128	1,024	1.024	0

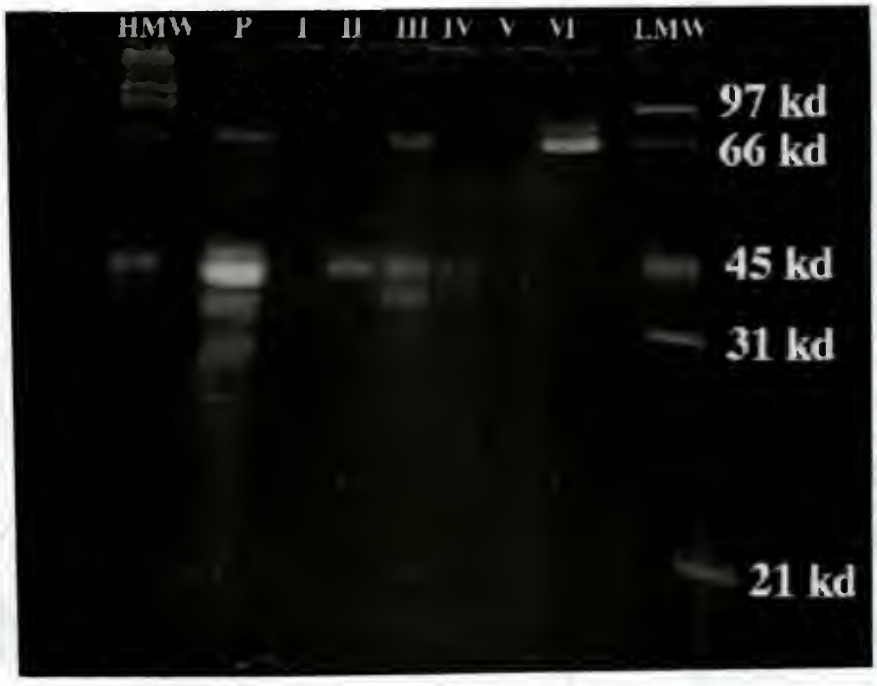


Figure 3-4. SDS-PAGE of fucose binding proteins eluting in various fractions from Sephacryl S-300. P is the parental affinity purified sample applied to the gel permeation column. Fraction II contains the highest hemagglutination titer.

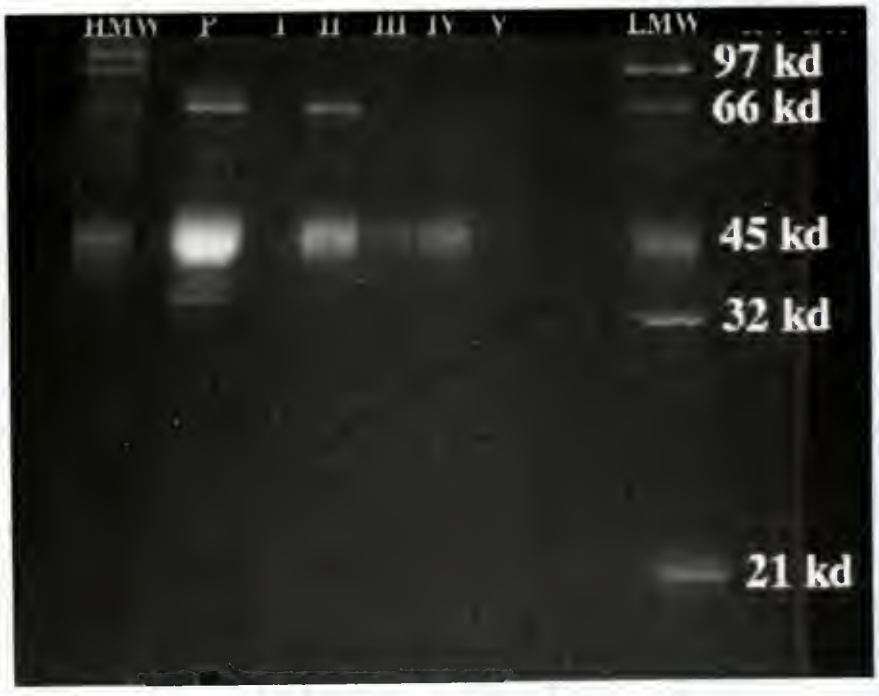


Figure 3-5. SDS-PAGE of galactose binding proteins eluting in various fractions from Sephacryl S-300. P is the parental affinity purified sample applied to the gel permeation column. Fraction IV contains the highest hemagglutination titer.

Table 3-3. Characteristics of fractions from A. gemmatalis lectin purification. Specific activity was calculated as titer/mg protein. Units were calculated as titer x volume.

Fraction	Volume	Protein mg/ml	Titer	Specific Activity	# units
whole hemolymph	3 x 5 ml	35.05	8192	233.4	122880
FE (gal column)	3 x 30 ml	9.48	2048	216	184320
1x gal	45.5 ml	0.0042	128	30476	5824
2x gal	12 ml	0.0017	256	150588	3072
1x fuc	18 ml	0.0419	2048	48878	36864
2x fuc	15 ml	0.0107	256	22756	3840
3x fuc	10.5 ml	0.0112	512	45714	5376

Table 3-4. Characteristics of reconstituted lyophilized preparations of lectin from A gemmatalis. Units were calculated as titer x volume.

Fraction	Reconstituted Volume	Units
2X gal FE	2 ml	4096
2X gal salt cut	1 ml	128
2X gal lectin	0.5 ml	16384
3X fuc FE	0.5 ml	128
3X fuc lectin	0.5 ml	8192

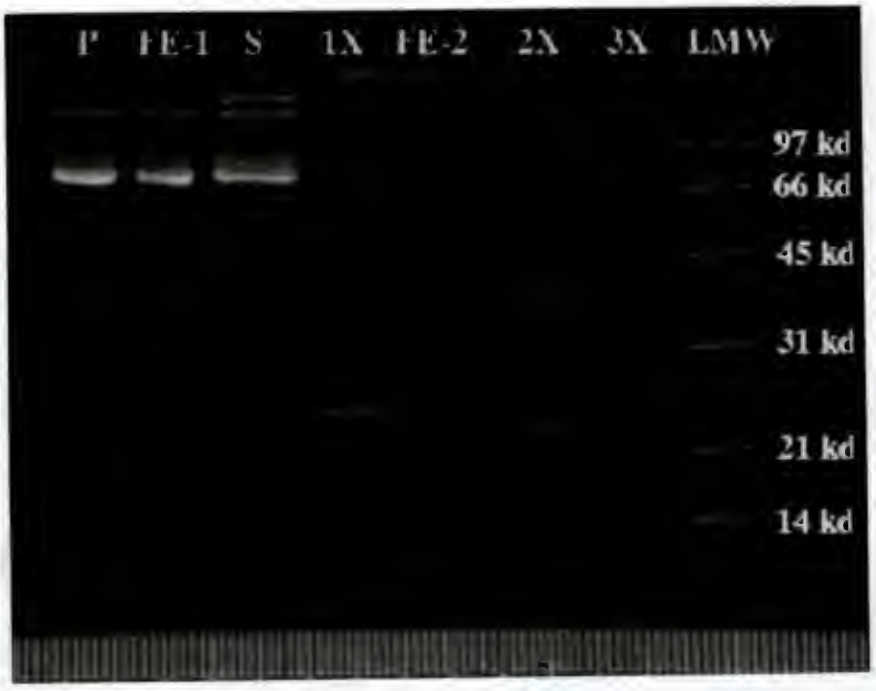


Figure 3-6. SDS-PAGE of fractions obtained during the purification of fucose binding proteins by double affinity chromatography. P is the parental hemolymph; FE is the first eluent; S is the NaCl cut; 1X, 2X and 3X are the purified fucose lectin fractions.

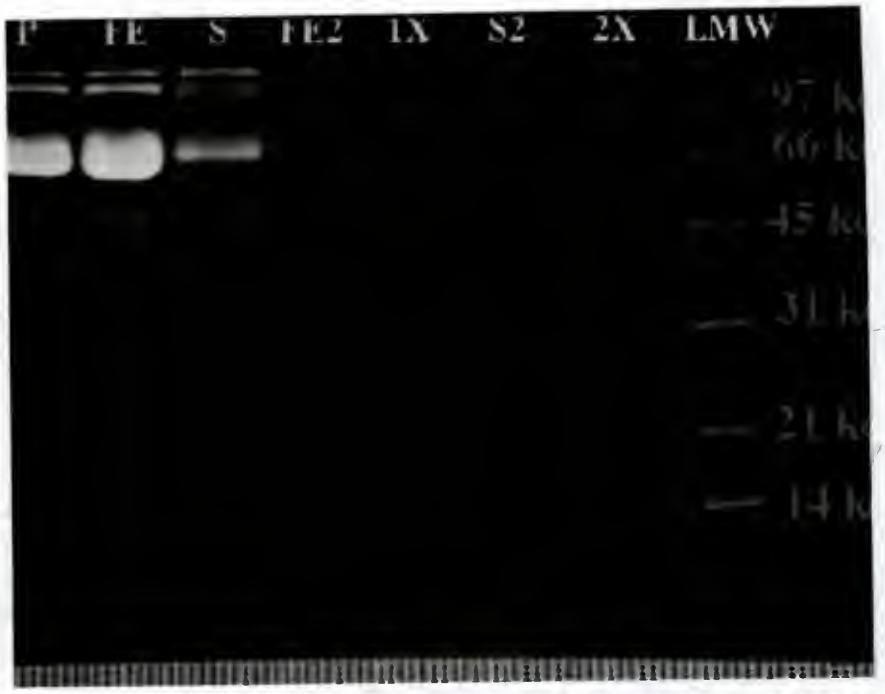


Figure 3-7. SDS-PAGE of fractions obtained during the purification of galactose binding proteins by double affinity chromatography. P is the parental hemolymph; FE is the first eluent; S is the NaCl cut; 1X and 2x are the purified galactose lectin fractions.

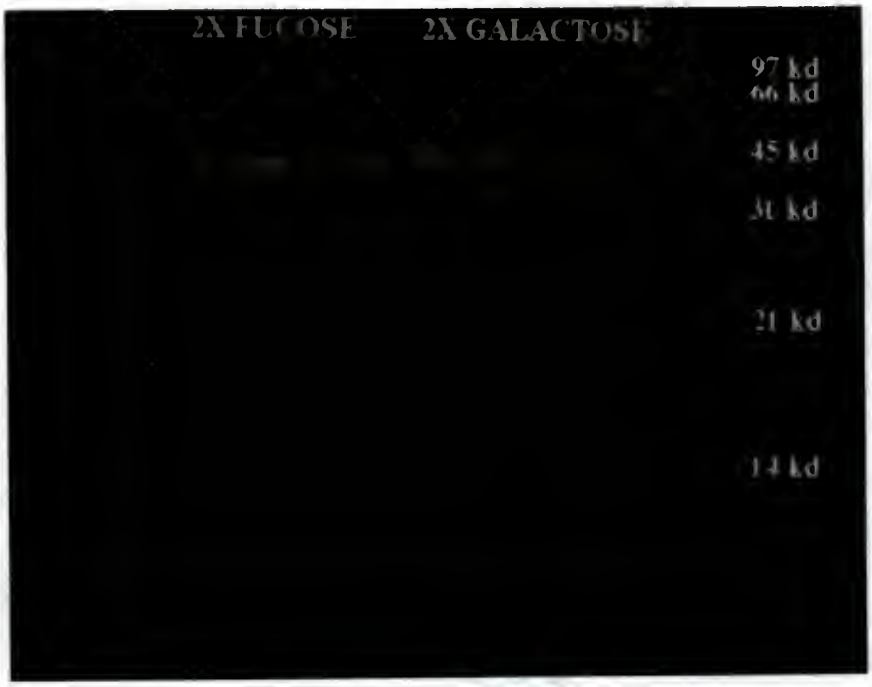


Figure 3-8. SDS-PAGE of 2X purified galactose and fucose lectins purified by double affinity chromatography.

Figure 3-8 shows the appearance of purified fractions used for antibody production and demonstrates inconsistency in purification, especially for the FBPs. As determined from assays of dialyzed fractions, the efficiency of purification was 6% as determined by addition of units from 2X gal lectin + 3X fucose lectin divided by apparent units in whole hemolymph. However, as shown in Table 3-4, when the purified fractions were lyophilized and reconstituted, there was an increase in the number of units recovered and the efficiency was 20% if the total number of units in whole hemolymph was used for the calculation. If the activity in the first eluent was used for the calculation, there was 13% recovery of activity. If all the activity recovered was considered, 23.5% of the activity was recovered in some form.

Discussion

This discussion will consider data presented in this chapter and in Appendix A. The information obtained from column chromatography (affinity chromatography and gel permeation chromatography) yielded insights into the A. gemmatalis lectin system. All of the gel permeation studies using BioGel 1.5A failed to effect separation of a lectin fraction and it was felt that the galactose-binding lectin might be adhering to the galactose-based matrix (agarose) thus causing elution over broad MW ranges. These

interactions were not overcome under any of the buffer conditions tested (presence of galactose in the buffer, high salt, EDTA, etc.) and the results from these experiments could be analyzed only after data from the double affinity purifications was obtained. The ability of the lectin to migrate successfully in polyacrylamide gels suggested that it did not stick to this resin. When the BioGel P 300 resin was used, the activity eluted in the void volume as a cohesive, impure peak. BioGel P has a nominal exclusion MW of 300 kd. Preliminary results from native gel electrophoresis had demonstrated that the native molecule of both GBPs and FBPs had an apparent MW in the 360 kd and 720 kd range (data not shown). This would indicate that the native molecule is at least an octamer and perhaps aggregates into a 16-mer or simply a large aggregate with no particular subunit composition. Finally, Sephacryl P-300 was chosen for use because it was polyacrylamide based and could allow for separation of molecules over a broad MW range (10 kd - 1,500 kd). Evidence suggested that the addition of galactose to the buffer might inhibit adherence of GBPs to the matrix and to other hemolymph proteins. This column provided critical information into the nature of the A. gemmatalis lectin system but since hemagglutinin activity did not elute in a narrow MW range on this gel and the column did not effect efficient purification of either

lectin, a double affinity purification was the method of choice.

As reported in Appendix A, small amounts of lectin can also be purified from normal hemolymph and with a mannose-agarose column. This supports the likelihood that one of the lectin components, probably the fucose lectin is a constitutive factor in the hemolymph. Dot blots (data not shown) probed with peroxidase labelled lectins have provided evidence that both 2X purified lectins bind to Con A and not to peanut agglutinin or wheat germ agglutinin indicating that the molecules are glycosylated and have glucose and/or mannose residues. The fucose lectin, with its weak mannose-binding capabilities may bind to the galactose lectin as well as to the mannosylated arylphorin. If this occurs, it would explain the difficulty in separating the component lectins from each other and from arylphorin. Arylphorin has been found to be the dominant serum protein in late instar lepidopteran larvae (Telfer et al., 1983; Haunerland and Bowers, 1986). Nonspecific aggregations of these molecules would also explain the inconsistent results obtained for purification protocols because seemingly minor inconsistencies in purification procedures, e.g. length of salt wash, could subsequently affect the purity of the molecules which eluted in the presence of the inhibiting sugar.

The purification efficiency data are of interest in that they corroborate other existing data which show that the galactose lectin has high specific activity. The fucose lectin does not have similarly high specific activity. During the course of purification, there was an increase in specific activity of 646 fold for the galactose lectin as compared to 195 fold for the fucose lectin. The yield of lectin is not good and most likely does not truly reflect the amount of total hemagglutinin (especially FBPs) present in the hemolymph. Some of the active molecules may elute in the salt wash and, although not present in sufficient quantity to give a detectable titer, are nonetheless nonspecifically stripped away from the galactose lectin. This could explain the dramatic loss in fucose activity during the course of purification.

Results shown in Tables 3-2 and 3-3 point out the fallacy of relying on HA titers for measuring activity. After lyophilization and reconstitution, there was a dramatic increase in activity most likely because the molecules had undergone self aggregation. If 184,320 units represent the total amount of fucose lectin, then 20% is recovered with one passage through fucose agarose, but less than 3% remains after 3X purification.

The amenability of the galactose lectin to purification is much greater than the fucose lectin. Although silver staining has not been performed, the

galactose lectin can apparently be purified to homogeneity or near homogeneity while the fucose lectin shows the presence of multiple bands even after 3X purification. As shown in Figure 3-6, some of the fucose lectin may exist as 22.5 kd subunits. However, a previous experiment in which 2X purified lectin preparations had been boiled in lysis buffer for up to 30 min failed to detect these low MW subunits and they may represent degradation products of the lectin.

In the hemolymph, the galactose lectin may exist bound to other hemolymph components which render it inactive (e. g. (Fraction II of galactose lectin as shown in Figure 3-3). This is evidenced in Figure 3-3 by comparing fractions II and IV. Whether results from minigels and large gels are completely compatible is also uncertain. Although fraction II (Figure 3-3) contains more protein, fraction IV has 16 times greater activity and apparently the barely detectable amount of lectin in fraction III represents the same amount of activity as in fraction II. However, the total activity of this preparation was 99.6% inhibitable by fucose in addition to being totally inhibited by galactose.

Another problem experienced during affinity purification was that at the absorbance sensitivity used to detect lectin, galactose also absorbs and frequently the elution of the galactose lectin was barely detectable. In addition, the fraction showed no HA activity while bound to

the galactose. Through experience, it was found that the void volume of 5 ml of packed resin was about 2.5 ml and the galactose lectin, which could barely be detected by monitoring UV absorbance and was inactive because it was bound to the galactose, would elute in approximately 8 ml.

CHAPTER 4

SUGAR INHIBITION PROFILES

Introduction

Traditionally, lectins have been partially categorized based on the monosaccharide or oligosaccharide which provides most effective hemagglutination inhibition (see Chapter 1). Previous work by Pendland and Boucias demonstrated that binding of the Anticarsia gemmatalis lectin to trypsinized rabbit RBC could be inhibited by lactose, D-galactose and L-fucose. The agglutination of human O erythrocytes, however, was inhibited solely by N-acetyl neuraminic acid. The diluting buffer was phosphate buffered saline (PBS).

During the course of affinity purification, it became apparent from hemagglutination inhibition (HI) studies (using HI method I) that L-fucose was a more potent inhibitor of hemagglutination (HA) than D-galactose and experiments were undertaken, initially using immune hemolymph and later using purified and partially purified lectin, not only to look at the inhibition profiles to categorize the lectins but also to try to understand how the lectins function in this insect. It was postulated that

inhibition studies might explain some of the difficulties experienced during purification. Additionally, studies were undertaken to ascertain if either lectin was blood group specific and thus useful as a diagnostic reagent.

Materials and Methods

Erythrocytes. Rabbit RBC were obtained locally or from Hazelton Research Products (Denver, PA). Human RBC were obtained as outdated material from the Blood Bank at the JH Miller Health Center in Gainesville, FL. Blood cells were washed several times in PBS, pH 7.2 and usually trypsinized prior to use according to the method of Novak et al. (1970). For use, the cells were counted with a hemacytometer and diluted to the appropriate concentration. For HA, a 2% solution (3×10^8 cells) was generally used. For inhibition studies, a 4% solution was usually used.

Hemagglutination Assay. For assay, serial twofold dilutions of hemolymph, lectin or other test material were made in V-bottom microtiter plates using either PBS or buffered insect saline (BIS) (Castro et al., 1987) as diluent. Frequently, it was desirable to conserve hemolymph or purified lectin and 10 μl of hemolymph were added to 70 μl diluent giving a starting dilution of 1:8. Then 50 μl were transferred to a well containing 50 μl diluent and subsequently, serial twofold dilutions made. In the first well, 30 μl of RBC were added. An equal volume (usually 50

μl) of erythrocytes was added to the following wells and after 1 hr incubation at room temperature, the plates were read. The plates were refrigerated and reread after several hours or overnight. A positive reaction appeared as a diffuse mat in the bottom of the well and a negative reaction appeared as a red dot. Positive and negative controls were included in each group of assays. The titer was expressed as the reciprocal of the highest dilution giving complete HA. One peculiarity of the test system was the presence of incomplete hemagglutination. These results were carefully and conservatively evaluated and will be discussed later.

Hemagglutination Inhibition (HI) Assay I. Sugars were usually obtained from Sigma Chemical Co. (St. Louis, MI) and were of reagent grade. Test sugars were prepared as 200 mM solutions in either PBS or BIS. Serial twofold dilutions of hemolymph or lectin were prepared using the sugar solution as diluent and after 1 hr at room temperature, an equal volume of a 2% solution of test RBC were added. After an additional incubation at room temperature for 1 hr, the plates were read. Plates were reread after several hours or overnight incubation at 4 C. Positive and negative controls were included with each group of assays. The titer was recorded as the reciprocal of the highest dilution giving complete inhibition.

Hemagglutination Inhibition (HI) Assay II. For this assay, the titer of the hemolymph, lectin or test substance was determined and considered one unit e.g. with a titer of 1024, a 1:1024 dilution yields one unit. For HI, four units were used e.g. a 1:256 dilution. Test monosaccharides were usually prepared at a concentration of 800 mM in BIS or occasionally in PBS. Other sugars such as disaccharides, relatively insoluble sugars or expensive sugar derivatives were prepared at a lower concentration. To the first well of the microtiter plate were added 50 µl of test sugar. In the second well 50 µl were added to 50 µl diluent and serial twofold dilutions made. To each well were added 50 µl of solution containing 4 units hemagglutinin. After an incubation of 1 hr at room temperature, a solution of 4% RBC was added. The HI assay was read after the 1 hr incubation and after refrigeration for several hours or overnight. Positive and negative controls were included with each assay and consisted of hemagglutinin + RBC, inhibiting sugar + RBC; diluent + RBC. In calculating the minimum inhibitory concentration (MIC), the dilution of stock solution allowing for dilution by hemagglutinin or diluent was recorded as the MIC. Since four units of added lectin contained adequate agglutinin, no dilution factor was considered. This was also true for the indicator system - the test RBC.

Results

Optimization of HA test. These results are shown in Table 4-1. The buffer giving consistently high titers was BIS and this was generally used for the assays. One to two per cent rabbit RBC were found to give optimum titers.

Hemagglutination inhibition studies using A. gemmatalis immune hemolymph and variously treated erythrocytes. Results are shown in Table 4-2 and illustrate the inconsistencies obtained with different RBC. All hemolymph samples were diluted in BIS. Trypsinized rabbit RBC showed greater inhibition by L-fucose; trypsinized human O RBC exhibited very high titers with no specificity; nontrypsinized rabbit RBC showed a markedly decreased titer and HA could be inhibited completely by galactose, L-fucose, lactose and moderately by glucose and mannose; nontrypsinized human 0 RBC were inhibited only by L-fucose.

Since the two dominant inhibitory monosaccharides were D-galactose and L-fucose, HI Assay II was used to test the ability of these sugars to inhibit HA of trypsinized rabbit RBC using immune hemolymph and either PBS or BIS as diluent. These results are shown in Table 4-3. The agglutinin best inhibited by fucose seemed to require Ca^{++} ions while the agglutinin best inhibited by galactose did not seem to require this divalent cation. These results suggested that A. gemmatalis immune hemolymph contained two component lectins which differ in sugar specificity.

Sugar inhibition profiles using HI-I. After twice purified lectins were obtained, inhibition tests were performed using 0.2 M galactose or fucose. Results are shown in Table 4-4. The purified galactose lectin was inhibited by both galactose and fucose while the partially purified fucose lectin was inhibited completely by fucose and slightly by galactose.

Sugar inhibition profiles using HI Assay II. These results are presented in Table 4-5. The two lectins exhibited different inhibition profiles. The twice purified galactose lectin was inhibited by all of the galactose derivatives and was inhibited best by the synthetic sugar m-nitrophenyl galactopyranoside. The galactose lectin showed no apparent anomeric specificity as indicated by equivalent inhibition by α and β lactose. The twice purified fucose lectin was inhibited best by L-fucose and the synthetic p-nitrophenyl fucopyranoside. Since the m-nitrophenyl galactopyranoside was relatively insoluble, the stock solution was 50 mM.

Block titrations. To resolve some of the apparent contradictions in data generated by the two HI assays, block titrations were set up using whole immune hemolymph, BIS as diluent, trypsinized RBC and either L-fucose or galactose as inhibitory sugar. As shown in Table 4-6, in the presence of L-fucose at concentrations of 400 mM, 200 mM and 100 mM, there was complete inhibition regardless of hemolymph

Table 4-1. Titers of whole hemolymph at various intervals following lectin induction and in the presence of various buffer systems.

	BIS+ 2%RBC	BIS+ 1%RBC	BIS+ 1%BSA+ 1%RBC	PBS+ 1%RBC	PBS+ 1%BSA+ 1%RBC
6 hr PI	256	2048	128	256	64
12 hr PI	512	4096	512	128	64
18 hr PI	2048	4096	1024	128	128
24 hr PI	2048	4096	1024	1024	128
36 hr PI	1024	512	128	64	128
48 hr PI	1024	2048	256	64	128

Table 4-2. Hemagglutination inhibition of erythrocytes by immune hemolymph of Anticarsia gemmatalis. Results are given as the titer in the presence of the test sugar. The numbers in parentheses are the number of wells reduction represented by this titer.

Trypsinized Rabbit RBC - Titer: 16,384		Trypsinized Human O RBC - Titer: 65,536	
+0.2 M galactose	2048 (3)	+0.2 M galactose	4096(4)
+0.2 M glucose	4096 (2)	+0.2 M glucose	8192(3)
+0.2 M mannose	4096 (2)	+0.2 M mannose	8192(3)
+0.2 M lactose	1024 (4)	+0.2 M lactose	8192(3)
+0.2 M L-fucose	64 (8)	+0.2 M L-fucose	8192(3)
Nontrypsinized rabbit RBC - Titer: 512		**Nontrypsinized Human O RBC -Titer: 512**	
+0.2 M galactose	<8 (>6)	+0.2 M galactose	512 (0)
+0.2 M glucose	64 (3)	+0.2 M glucose	512 (0)
+0.2 M mannose	128 (2)	+0.2 M mannose	512 (0)
+0.2 M lactose	8 (6)	+0.2 M lactose	512 (0)
+0.2 M L-fucose	8 (6)	+0.2 M L-fucose	64 (3)
+0.4 M L-fucose	<8 (>6)	+0.4 M L-fucose	64 (3)

Table 4-3. Hemagglutination inhibition of trypsinized rabbit erythrocytes exhibited by four units agglutinin using PBS or BIS as buffer.

Inhibition by	HA titer (PBS) 512 (4 units in PBS = 1:128 dilution)	HA titer (BIS) 4096 (4 units in BIS = 1:1024 dilution)
fucose	50 mM fucose (8.2 mg/ml)	6.25 mM fucose (1.02 mg/ml)
galactose	3.1 mM galactose (0.56 mg/ml)	400 mM galactose (72.08 mg/ml)

Table 4-4. Hemagglutination inhibition of trypsinized rabbit erythrocytes by 0.2 M sugar of twice purified lectins from Anticarsia gemmatalis.

Inhibition by	2X galactose lectin Titer: 128,000	2X fucose lectin Titer:32,000
0.2M galactose	<16	4096
0.2M fucose	32	<16

Table 4-5. Sugar inhibition profiles of twice purified galactose and fucose Anticarsia gemmatalis lectin using HI-II.

Inhibiting sugar	concentration (mM) to inhibit	
	2x galactose lectin	2x fucose lectin
D-galactose	0.39	>400
L-fucose	25.00	12.5
D-mannose	>400	>100
D-glucose	>400	>400
D-fucose	0.78	>400
trehalose	>200	>400
α-lactose	0.39	>200
β-lactose	0.39	>200
p-nitrophenyl gal	0.78	100
m-nitrophenyl gal	0.10	>25
p-nitrophenyl fuc	>200	25

Table 4-6. Block titration of serial twofold dilutions of whole immune hemolymph and serial twofold dilutions of L-fucose in BIS. Positive hemagglutination is designated as (+); nonhemagglutination is designated as (-); slightly positive hemagglutination is designated as (±).

	Concentration of L-fucose											
Diln. HL	4^{-1}	2^{-1}	1^{-1}	5^{-2}	2.5^{-2}	1.3^{-2}	6.3^{-3}	3.1^{-3}	1.6^{-3}	7.8^{-4}	3.9^{-4}	1.9^{-4}
8	-	-	-	+	+	+	+	+	+	+	+	+
16	-	-	-	-	+	+	+	+	+	+	+	+
32	-	-	±	+	+	+	+	+	+	+	+	+
64	-	-	-	±	+	+	+	+	+	+	+	+
128	-	-	-	-	+	+	+	+	+	+	+	+
256	-	-	-	-	-	±	+	+	+	+	+	+
512	-	-	-	-	-	-	+	+	+	+	+	+
1024	-	-	-	-	-	-	-	+	+	+	+	+
2048	-	-	-	-	-	-	-	-	-	+	+	+
4096	-	-	-	-	-	-	-	-	-	-	-	-
8192	-	-	-	-	-	-	-	-	-	-	-	-
16384	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-7. Block titration of serial twofold dilutions of whole immune hemolymph and serial twofold dilutions of D-galactose in BIS. Positive hemagglutination is designated as (+); nonhemagglutination is designated as (-); slightly positive hemagglutination is designated as (±).

	Concentration of D-galactose											
Diln. HL	4^{-1}	2^{-1}	1^{-1}	5^{-2}	2.5^{-2}	1.3^{-2}	6.3^{-3}	3.1^{-3}	1.6^{-3}	7.8^{-4}	3.9^{-4}	1.9^{-4}
8	-	-	-	-	-	-	-	-	+	+	+	+
16	-	-	-	-	-	-	-	-	+	+	+	+
32	-	-	-	-	-	-	-	+	+	+	+	+
64	-	+	+	+	+	+	+	+	+	+	+	+
128	+	+	+	+	+	+	+	+	+	+	+	+
256	+	+	+	+	+	+	+	+	+	+	+	+
512	±	+	+	+	+	+	+	+	+	+	+	+
1024	-	+	+	+	+	+	+	+	+	+	+	+
2048	-	+	+	+	+	+	+	+	+	+	+	+
4096	-	-	-	-	-	-	-	-	-	-	-	-
8192	-	-	-	-	-	-	-	-	-	-	-	-
16384	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-8. Hemagglutination titers of nontrypsinized human erythrocytes.

	Normal HL	Immune HL	2X galactose lectin	2X fucose lectin
Human A 1	64	512	<8	2048
Human A 2	128	512	<8	1024
Human B	64	512	<8	1024
Human O	128	512	<8	1024

Table 4-9. Hemagglutination titers of trypsinized human erythrocytes.

	Normal HL	Immune HL	2X gal	2X fuc
Human A 1	2048	32,768	64	32,768
Human A 2	1024	131,072	64	16,384
Human B	1024	4096	64	32,768
Human O	1024	32,768	64	4096

concentration. Starting at 0.05 mM, the inhibition was proportional to the dilution. As shown in Table 4-7, when galactose was used as the inhibitory sugar, there was no complete inhibition regardless of hemolymph concentration even at the 400 mM level. In the presence of high hemolymph concentrations (1:8; 1:16; 1:32), there was complete inhibition in the presence of sugar concentrations as low as 3.125 mM.

ABO blood group specificity studies. The results from these assays are shown in Tables 4-8 and 4-9. Normal hemolymph showed limited ability to agglutinate nontrypsinized human RBC and although trypsin treatment increased the HA titer substantially, the erythrocytes were agglutinated regardless of ABO blood type. Immune hemolymph showed an increased titer compared to normal hemolymph and trypsin treatment dramatically increased HA titer. Typsinized type A 2 cells were agglutinated readily but other blood types showed high titers. The purified galactose lectin showed no hemagglutinin activity towards nontrypsinized human RBC of known ABO specificity and very slight hemagglutinin activity towards trypsinized human ABO blood groups. The twice purified fucose lectin agglutinated human erythrocytes of all types and trypsin treatment increased HA titer.

Discussion

This series of experiments provided information on several aspects of the A. gemmatalis lectin system. Initial findings demonstrated that the presence of the divalent cation calcium improved hemagglutination titers (Table 4-1). When twice purified fucose lectin was obtained and tested, it was found that the absence of Ca^{++} ions resulted in complete loss of hemagglutinating activity (data not shown). The same was not true for the twice purified galactose lectin. This could explain why Pendland and Boucias (1986) found no inhibition of human O cells by L-fucose. It is not known why the concentration of Ca^{++} in whole hemolymph might not be adequate for hemagglutination. Unfortunately, the ion composition of A. gemmatalis hemolymph has yet to be researched. Cohen and Patana (1982) and Bindokas and Adams (1987) have determined various ion concentrations in insects. In fifth instar larvae of the noctuid Spodoptera exigua, the calcium concentration was found to be 8 mM which should be adequate for HA since the calcium concentration in BIS is only 1 mM. Cohen and Patana (1982) also analyzed starved larvae and were able to show that the calcium concentration decreased to 2 mM in these insects. Fungi like Nomuraea rileyi are thought to kill their host through nutrient depletion (McCoy et al., 1989) and perhaps this occurs in Anticarsia and the concomitant calcium depletion renders the endogenous fucose lectin inactive.

The data in Table 4-2 shows the effects of trypsin treatment on rabbit and human type O cells. For both trypsinized rabbit RBC and nontrypsinized human O cells, fucose was the dominant inhibitory sugar. Nontrypsinized rabbit RBC showed a great decrease in agglutinability but were inhibited equally well by galactose, fucose and lactose. Conversely, trypsinized human O cells exhibited a greatly elevated titer but were inhibited quite nonspecifically by all sugars tested. The reasons for this are unclear as the purified galactose lectin shows limited ability to agglutinate trypsinized Human O cells (Tables 4-8 and 4-9).

As shown in Table 4-3, using HI-II, immune hemolymph in the presence of BIS was best inhibited by fucose. In the presence of PBS, and at a much higher concentration, galactose was the best inhibitor. We have prepared monoclonal antibodies against the galactose lectin but initial studies using Enzyme Linked Immunosorbent Assay (ELISA) (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971) have been unable to distinguish between the galactose-inhibitable and fucose-inhibitable components suggesting that they possess common epitopes. Thus, the relative concentrations of the two component lectins in the whole hemolymph have yet to be determined and future work should be directed towards quantitatively partitioning the system. With suitable inhibition protocols and ELISA, these

experiments should be feasible. In general, the assay using PBS probably reflected the galactose lectin only while the BIS assay reflected activity of both lectins. These data can be used to interpret some results shown in Table 4-1. If titer in PBS can be considered an index of galactose lectin activity, the galactose lectin reaches peak levels at 24 hr post injection.

It would also be interesting to test normal hemolymph for the presence of the component lectins by ELISA because purification of small quantities of lectin from hemolymph of nonchallenged insects has been effected (see Appendix A) and normal hemolymph has the ability to agglutinate human erythrocytes. As alluded to in the materials and methods section, the HA results from normal serum were extremely difficult to interpret because of the presence of heavy agglutination which either formed a prozone and appeared negative or moderate agglutination that appeared as a ± reaction. This occurred using both PBS and BIS and the addition of 1 mM Mg^{++} to BIS also did not eliminate the problem (data not shown).

Although partial purification of the component lectins has been effected, knowledge of the native molecular structure is lacking. The native lectin appears to be an octamer, but the subunit composition is unknown. Each component of the system retains activity after purification but the biological function of the molecule may require

interaction of the two components. The twice purified fucose lectin most likely contains some contaminating galactose lectin and perhaps the galactose lectin contains residual fucose lectin. It would be helpful if there were known prototype lectins similar to those in the Anticarsia gemmatalis lectin system. The isolectins from Bandeiraea simplicifolia have subunits of similar MW and specificity (galactose and N-acetyl galactosamine). Superficially, the Hyalophora cecropia lectin system appears similar to the B. simplicifolia lectins but the A subunits exhibit specificity for galactose and N-acetyl galactosamine while the specificity of the B subunits is unknown and may not even be directed against a carbohydrate (Castro et al., 1987). Although a few lectins have been reported to show crossreactivity with galactose and fucose, this phenomenon does not appear to be common but could be explained in our system if there existed a single lectin with two distinct binding sites which recognized an oligosaccharide with both galactose and fucose moieties. Lectins with two distinct binding sites per subunit have not been described. The sugar inhibition studies demonstrated that the galactose lectin was 32 times more sensitive to inhibition by galactose and galactose-related sugars than was the fucose lectin to L-fucose. This was also shown by differences in specific activity as discussed in Chapter 3. The twice

purified lectin was inhibited by m-nitrophenyl galactose > D-galactose = α-lactose = β-lactose > D-fucose>> L-fucose. By the criterion of Gallagher (1984), the fucose lectin would be a borderline candidate for classification as a monosaccharide inhibitable lectin and perhaps it recognizes oligosaccharides. Inhibition studies using oligosaccharides have yet to be conducted. The ability of L-fucose to inhibit the galactose lectin can be explained in several ways. The most obvious is that there is indeed cross reactivity and although less efficient than the sugars closely related to D-galactose, it can recognize the L-configuration and bind. Similar results were obtained when constant concentration of sugar (0.2 M) was used to inhibit twice purified lectin (see Table 4-3). Results from the block titration (Table 3-9), however, suggest that such a potent preparation of lectin (titer 128,000) should require higher concentrations of sugar to achieve this level of inhibition. Sigma Chemical Co. purchases their L-fucose from another source and does not analyze for optical purity so this sugar could contain from 1-3% D-fucose (personal conversation with representative from Sigma). This small percentage of D-fucose could account for results obtained in Table 4-5 but not necessarily those in Table 4-4. It is also interesting that there is no apparent anomeric specificity exhibited by the galactose lectin as both α- and β-lactose were equally effective in inhibiting agglutination

by the galactose lectin. Evidence shown in Table 4-4 suggested that in the presence of high concentrations of many sugars, which may simulate locally high sugar concentrations on microbes or other self glycoproteins or glycolipids, binding may occur to sugars otherwise nominally not crossreactive, e.g. mannose.

The discrepancies in results between HI-I and HI-II were analyzed by block titrations (Tables 4-6 and 4-7) and show some interesting properties of the lectins. In the presence of high concentrations of sugar, fucose was the better inhibitor and as concentration of lectin and sugar decreased the two components continued to interact and hemagglutination was inhibited. The galactose lectin, on the other hand, was sensitive to low concentrations of galactose but required high concentrations of hemolymph for inhibition. Again, this may be due to gross differences in the concentrations of the two component lectins in immune hemolymph--the fucose lectin being present at much higher concentrations. At high concentrations of lectin, higher concentrations of inhibitor are required. As the concentration of lectin decreases, the concentration of sugar required for inhibition decreases proportionally. Conversely, the galactose lectin occurs in low concentrations but can be inhibited by minute quantities of sugar. These titrations also point out problems in interpreting results from HI tests. Using HI method I at a

concentration of 200 mM, fucose appeared to be the dominant inhibitory sugar compared to 200 mM galactose. Using HI method II (4 units of hemagglutinin e. g., 512 dilution), fucose also appeared to be the dominant inhibitory sugar. It would be instructive to conduct these studies using equivalent concentrations of the purified lectin components but sufficient quantities of purified lectin are unavailable at this time. It would also be interesting to analyze the lectins by quantitative precipitin and equilibrium dialysis methods to see if these results reflect differences in the number of binding sites, binding affinity or binding avidity.

How these lectins function in the insect is unknown. Perhaps the lectins act in concert and the "endogenous" fucose lectin detects high concentrations of sugar, e.g. locally high concentrations on surface of fungal cells or nonself glycoproteins and glycolipids. After induction, the galactose binding lectin is produced and the system is more sensitive to minimal concentrations of galactose residues. This might be analogous to the class switching observed in vertebrate immune systems where the large IgM molecules are replaced by smaller, more efficient IgG molecules which continue to undergo affinity maturation.

A protein of MW 66-70 kd commonly co-purified with the agglutinins (see Chapter 3). The major hemolymph protein in late instar larvae of other lepidopterans is arylphorin

(Telfer et al., 1983; Haunerland and Bowers, 1986; Karpells et al., 1990). In the insects studied (Heliothis zea, Manduca sexta, Hyalophora cecropia, and Lymantria dispar) the arylphorins are antigenically similar and glycosylated. Both the H.zea and H. cecropia proteins contain glucosamine and mannose in a 1:5 molar ratio. In H. zea, there is 2.5% carbohydrate present. One of the lectins (most likely the fucose lectin) in the Anticarsia system could conceivably interact with the mannose residues on this protein as small quantities of lectin have been partially purified using a mannose-agarose affinity column (data not shown). This remains to be tested in the near future.

Since fucose lectins with blood group specificity are relatively rare, studies were undertaken to determine if either of the lectins might be useful as a diagnostic reagent. As shown in Table 4-8 and 4-9, the galactose lectin showed little ability to agglutinate any of the blood types (even the nominally galactose specific Type B) and the nominal fucose lectin readily agglutinated all blood types. This difference may also reflect the ability of the fucose lectin to recognize a species distinct nonterminal oligosaccharide sequence found on all blood types and the inability of the galactose lectin to recognize terminal monosaccharides on any of the human blood types. The lack of apparent anomeric specificity exhibited by the galactose lectin may also explain its inability to discriminate α-

lactose from β-lactose. Perhaps the cells were fragile due to age and trypsin treatment rendered them very susceptible to agglutination in the presence of the fucose lectin. It would be valuable to repeat these tests with fresh human cells to clearly rule out whether either lectin is blood group specific.

It is interesting that many insect hemolymph lectins show galactose specificity while the target erythrocytes used are variable. In the human ABO group, galactose is considered the inhibitory monosaccharide for blood group B but, as demonstrated in these experiments, the Anticarsia galactose lectin does not recognize it. Unfortunately, the surface carbohydrate composition of rabbit RBC has not been well-characterized and the target mono- or oligosaccharide recognized by the Anticarsia lectins remains unknown. Komano et al.,1980, used sheep RBC as their target cells and these cells possess a dominant antigen (the lipopolysaccharide Forssman antigen) which the rabbit RBC do not possess. Castro et al., 1987, were unable to inhibit agglutination of rabbit erythrocytes with galactose using a galactose-specific lectin and perhaps the RBC from the rabbit were anomalous. The effect of the trypsin treatment is also unknown but may clear away some peptides which might interfere with effective cross-linking of lectin molecules. However, results in Table 4-2 showed that agglutination of nontrypsinized rabbit RBC could be inhibited by galactose,

fucose and lactose. Stebbins and Hapner (1985) used neuraminidase treated human erythrocytes. This treatment removes the terminal sialic acid residues from erythrocytes.

CHAPTER 5

OPSONIC PROPERTIES OF *Anticarsia gemmatalis* HEMOLYMPH

Introduction

Agglutinins of vertebrate erythrocytes, protozoan parasites and bacteria have been reported to occur in insects (see Chapter 1 for review) but the functions of these molecules are obscure. In vertebrates, nonimmune serum factors are required for effective binding and ingestion of microorganisms by phagocytes. These factors have been termed opsonins (from the Greek "opsono," to prepare food for). Because of their carbohydrate binding ability which may mediate interaction with oligosaccharides on cell membranes and the cell walls of microorganisms, lectins, including those of insect origin, have been investigated for possible opsonic function. Ratcliffe and Rowley (1983) searched for and failed to find an opsonic function for agglutinins from various insects. Rather, they suggested these molecules act as recognition factors which are activated by cell wall constituents of microorganisms. Hapner *et al*. (1987) also failed to find an opsonic function for the galactose lectin of *Melanoplus* spp. but these investigators used a microbial agent lacking exposed

galactose residues (the microsporidian pathogen Nosema locustae) in an attempt to demonstrate a function for a galactose-binding lectin. Pendland et al. (1988) were able to demonstrate an opsonic function for an insect agglutinin using the galactose lectin from larval Spodoptera exigua and the fungus Paecilomyces farinosus. This fungus had been investigated by Pendland and Boucias (1986b) and was shown to possess exposed galactose residues on its cell wall. Both in vitro studies using hemocyte monolayers and in vivo clearance studies with S. exigua demonstrated that opsonization enhanced phagocytosis and clearance of P. farinosus. Subsequent to this initial finding, other investigators reported that this function also existed in other insects. Drif and Brehélin (1989) found that agglutinins present in hemolymph of Locusta migratoria were able to act as opsonins of sheep erythrocytes. Lackie and Vasta (1988) found that a similar function existed in Periplaneta americana.

Pendland et al. (1988) also speculated that both specific and nonspecific opsonic factors existed in S. exigua. The phagocytic granulocytes possessed galactose residues and thus could cross-link with P. farinosus opsonized with the galactose lectin. The plasmatocytes (which are also phagocytic in this insect) lacked exposed galactose residues, and it was postulated that phagocytosis by plasmatocytes was mediated by the nonspecific factors.

Fryer and Bayne (1989) have found that two types of recognition occur in the snail Biomphalaria glabrata. The plasma opsonin recognizes mannose while the hemocyte receptor recognizes β-1,3 glucan. In addition, the phagocytic hemocytes are thought to nonspecifically recognize and phagocytose nonself. Recently, Maťha et al. (1990a, 1990b) have reported the existence of a β-1,3 glucan lectin in Galleria melonella hemocytes. Investigators had previously failed to find a hemolymph lectin in this insect. Since β-1,3 glucans are common constituents of fungal cell walls and are known to activate prophenoloxidase in insects (Ashida et al., 1982; see Sugumaran (1990) for review), this finding suggests that insect defense mechanisms are interrelated. A plasma β-1,3 glucan receptor has been reported in Bombyx mori (Yoshida et al., 1986) and has been suggested to occur in Blaberus craniifer (Söderhäll et al., 1988).

Many intriguing questions still exist as to the function of insect lectins. The experiments described in this chapter were undertaken to look for an opsonic role for Anticarsia gemmatalis hemolymph.

Materials and Methods

Insects. Larval A. gemmatalis were reared as previously described and late sixth instar larvae were used

for the experiment. After injection, insects were housed individually in 24 well plates.

Preparation of inoculum. Hyphal bodies of Nomuraea rileyi were maintained on Sabouraud Maltose Yeast agar and prepared as described in Chapter 2. A culture of P. farinosus was prepared in Sabouraud Dextrose Yeast broth. Prior to use, contaminating mycelia were removed by filtration through Miracloth (Calbiochem, LaJolla, CA) and the blastospores washed and prepared as described for N. rileyi. After enumeration with a hemacytometer, the cells were diluted to 1 x 10^8 per ml and 500 μl aliquots dispensed into microcentrifuge tubes. The cells were pelleted at 10,000 x g for 5 min, the supernatant removed and 1 ml of either buffered insect saline (BIS), immune or normal A. gemmatalis hemolymph added to the tube. The preparation of immune hemolymph has been described in Chapter 3 and also appears in Appendix B. Normal hemolymph was collected from untreated sixth instar larva in the same manner as described for immune hemolymph. After incubation on a rocker platform for 2 hr at room temperature, the cells were pelleted, washed in BIS, resuspended in 500 μl BIS and injected into the insects as previously described. Hemolymph samples were obtained at various intervals after injection by puncturing a proleg and a 10 μl sample was diluted with 90 μl BIS containing phenylthiourea (PTU). The numbers of fungal cells and hemocytes were determined with a hemacytometer.

Experimental design. Four groups of 20 insects were used for each treatment and groups of five insects were bled at 15 min, 30 min, 60 min and 120 min intervals after injection. The number of hyphal bodies or blastospores and the total hemocyte count (THC) was determined for each sample with a hemacytometer. No attempt was made to differentiate hemocyte types. One group of 20 insects from each treatment was selected for an analysis of hemagglutination (HA) activity. After the numbers of hemocytes and fungal elements in the sample were determined, these cells were pelleted by centrifugation and an HA assay performed on the cell-free diluted hemolymph as previously described. For comparison of HA titers, twelve untreated late sixth instar larvae were bled and the sample prepared as described above. Normal hemocyte counts were also determined from untreated larvae.

Hemagglutination (HA) assay. This assay has been previously described in detail. Hemolymph samples were collected as 1:10 dilutions in BIS and due to the limited volume available, the starting dilution was 1:20. Serial twofold dilutions were prepared in BIS and an equal volume of 2% trypsinized rabbit RBC added as previously described. Titers were evaluated an incubation of 1 hr at room temperature and after refrigeration for several hr or overnight.

Results

The results from the opsonization experiments are shown in Figures 5-1 and 5-2. The results presented are expressed as number of fungal elements/ml hemolymph. Blastospores known to possess exposed galactose residues were cleared from the hemolymph while the hyphal bodies of N. rileyi were not cleared. When analyzed statistically using Student's t test (Steel and Torrie, 1960), several treatments were found to be significant. With P. farinosus-treated insects, these treatments were: 30 min - BIS/Immune $p=0.044$; Normal/Immune $p=0.021$; 60 min - BIS/Immune $p=0.033$; 120 min - BIS/Normal $p=0.015$. When the N. rileyi treated insects were analyzed, only the Normal/Immune treatments at 60 min ($p=0.021$) showed significant difference.

After opsonizing substances in normal or immune hemolymph were allowed to be absorbed by the fungal cells, the hemolymph samples (normal and immune) were analyzed for HA activity. Opsonization of P. farinosus removed a half well of HA activity from immune hemolymph (reduction in titer from 8192 to 8192±) while the opsonization of N. rileyi failed to remove even this amount of activity.

Total hemocyte counts of insects in the various treatments are shown in Table 5-1 and expressed as number of hemocytes/ml. Compared with the average THCs from nontreated insects, THC from insects in the various treatments appeared to show an initial decrease followed by

return to normal levels over the course of the experiment. However, the range in numbers of hemocytes from untreated insects is so varied that these results are not significant and are presented to show there is no dramatic increase or decrease in total hemocytes following treatment.

The HA profiles of normal insects are shown in Figure 5-3 to allow for comparison with HA profiles obtained from insects in the various treatment groups (Figure 5-4 and 5-5). For analysis of hemagglutinin in control hemolymph, samples were collected from 12 insects. The black areas represent a negative or an apparently negative prozone both of which appear as a distinct red pellet in the bottom of the well. In this control group, there was no prozone phenomenon (an apparently negative reaction) and hemolymph from three insects showed no hemagglutinin activity. The gray shaded areas represent ragged, incomplete hemagglutination which will be interpreted as interaction of nonspecific and specific hemagglutinating factors. Hemolymph from two insects showed only ragged hemagglutination. The white areas represent typical diffuse even mat indicative of positive hemagglutination. Samples from seven insects showed an intermediate pattern--several wells of ragged hemagglutination ("titer" 160-320) followed by one or several wells of positive hemagglutination (titer 320-1280). The intent of the three dimensional representation is to convey that there may be several

factors involved in hemagglutination. A negative reaction is factored into the forwardmost row of blocks. The central row represents the amount of incomplete activity and the back row depicts the amount of true hemagglutinin present. If the three dimensionally depicted components were presented in a collapsed format, the components would appear as depicted in Figures 5-4 and 5-5.

In Figures 5-4 and 5-5, the hemagglutination was interpreted in the same manner as described above but the types of hemagglutination are stacked to facilitate presentation of the data. As shown in Figures 5-4, only in the insects challenged with BIS treated N. rileyi does the pattern seem to correlate with that of normal hemolymph both in intensity of response and appearance of the specific and nonspecific hemagglutinin. Both specific and nonspecific opsonins are removed from the hemolymph of insects injected with N. rileyi opsonized with either immune or normal hemolymph. In Figures 5-5, insects challenged with BIS-treated P. farinosus, show decreased levels of agglutinin, insects challenged with normal hemolymph opsonized P. farinosus show decreased titers of specific and nonspecific hemagglutination and insects challenged with immune hemolymph opsonized P. farinosus show a remarkable depletion of all types of hemagglutination up to 120 min post injection.

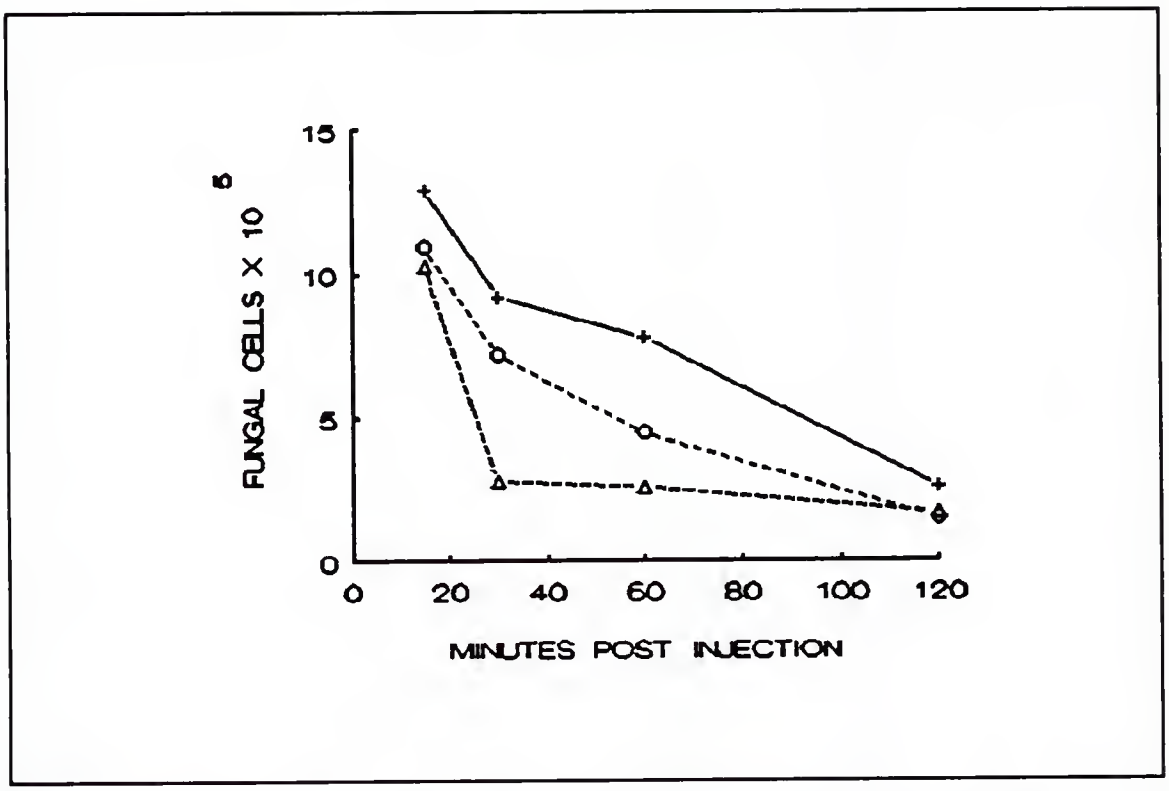


Figure 5-1. Clearance of opsonized and nonopsonized P. farinosus from hemolymph of A. gemmatalis. Δ - immune opsonized; o - normal opsonized; + - BIS opsonized

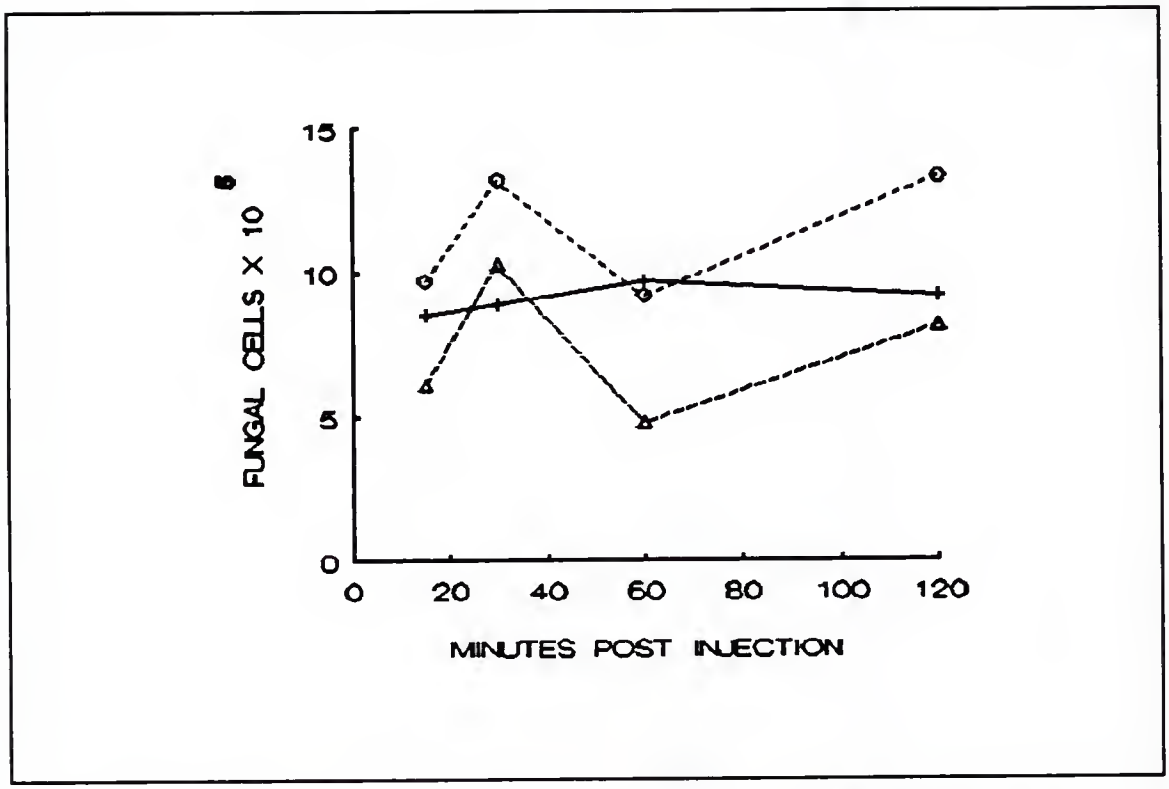


Figure 5-2. Clearance of opsonized and nonopsonized N. rileyi from hemolymph of A. gemmatalis. Δ - immune opsonized; o - normal opsonized; + - BIS opsonized

Table 5-1. Number of circulating hemocytes (x 10^6) at various time intervals following treatment with opsonized and nonopsonized fungi.

Treatment	Time after treatment (in min) 15	30	60	120
BIS/*P.farinosus*	16.3±7.4	16.7±5.5	19.5±5.0	27.5±7.7
Normal/*P.farinosus*	15.7±4.0	21.3±8.0	25.3±5.9	24.5±7.7
Immune/*P.farinosus*	16.6±5.2	22.6±6.6	25.2±5.1	21.7±5.2
BIS/*N.rileyi*	18.1±5.7	19.6±5.5	25.7±7.5	32.0±11.2
Normal/*N.rileyi*	17.6±10.4	20.4±6.8	25.1±10.5	18.8±9.3
Immune/*N.rileyi*	14.0±5.6	13.6±6.9	15.3±5.7	10.8±4.5

Normal hemocyte levels: 23.2±12.8 x 10^6

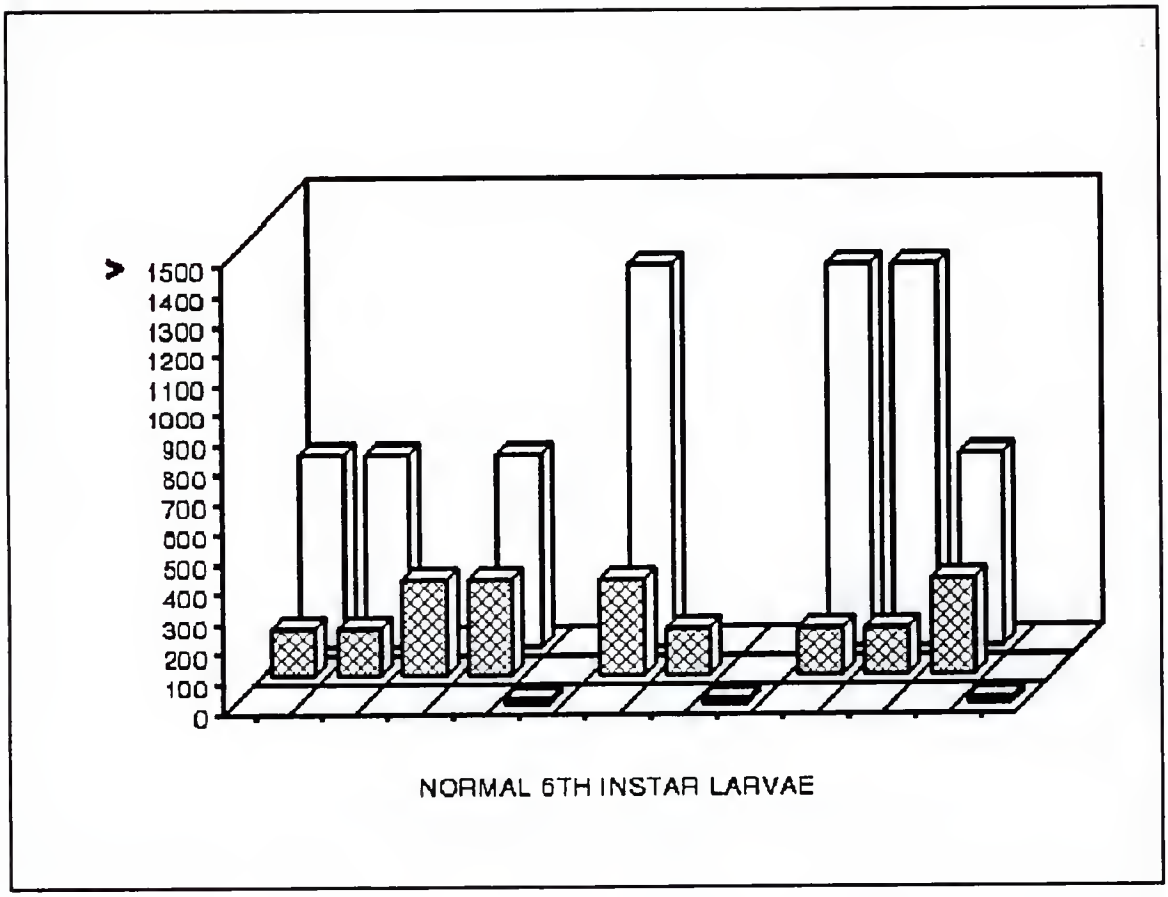
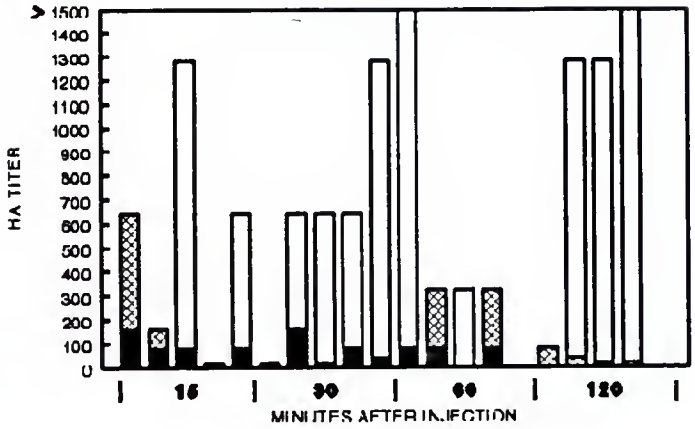
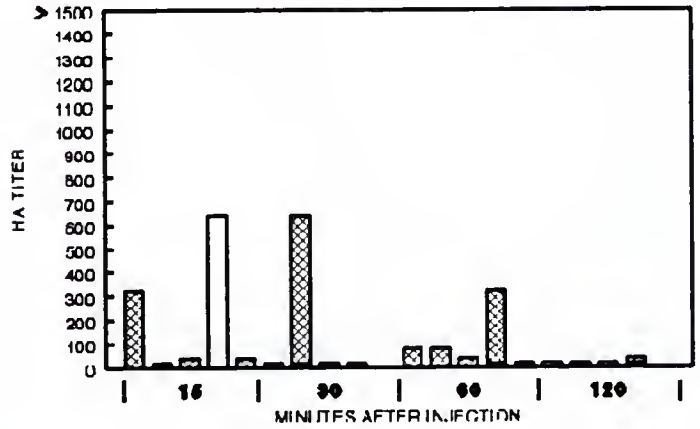


Figure 5-3. Individual hemagglutination profiles of normal hemolymph samples from untreated 6th instar larval Anticarsia gemmatalis. The black blocks (insects 5,8 and 12) represent a negative reaction; the shaded blocks in the center row represent the amount of incomplete, ragged hemagglutination; the white blocks represent the amount of complete hemagglutination.

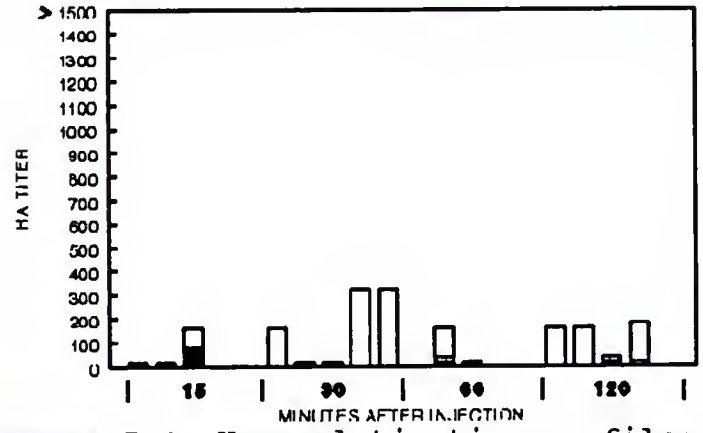
N. rileyi opsonized with BIS



N. rileyi opsonized with normal hemolymph

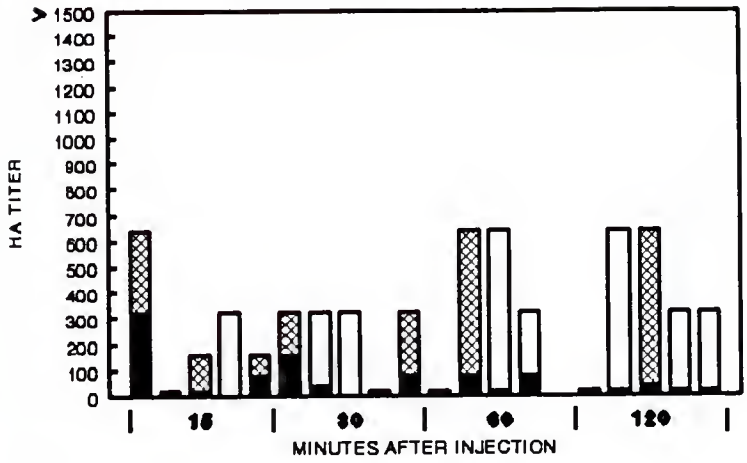


N. rileyi opsonized with immune hemolymph

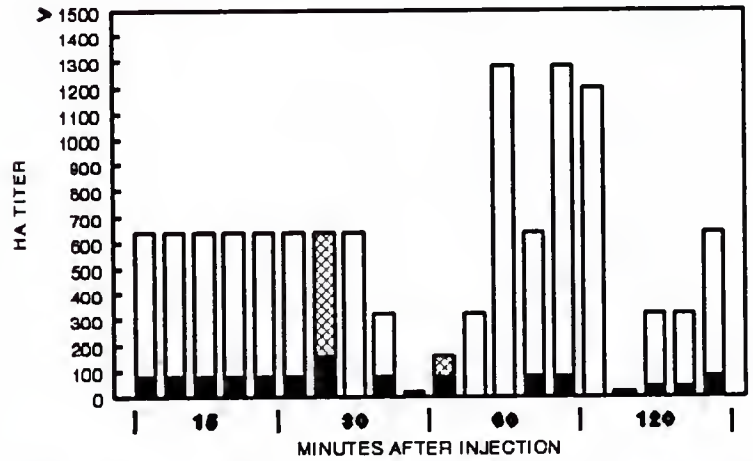


Figures 5-4. Hemagglutination profiles of hemolymph from A. gemmatalis treated with opsonized and nonopsonized N. rileyi.

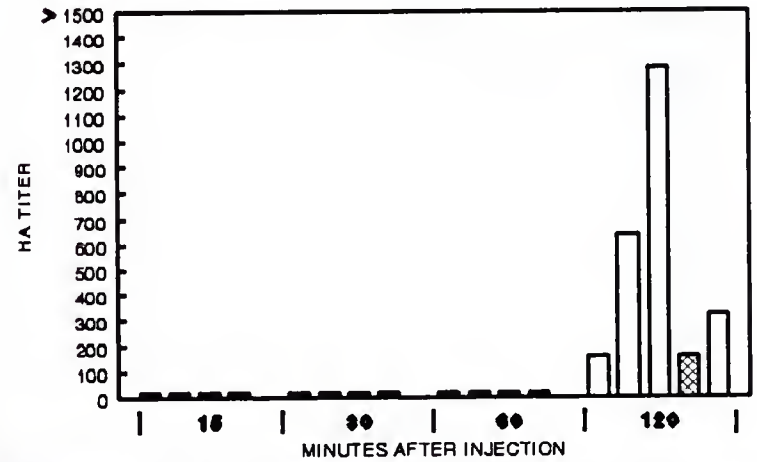
P. farinosus opsonized with BIS



P. farinosus opsonized with normal hemolymph



P. farinosus opsonized with immune hemolymph



Figures 5-5. Hemagglutination profiles of hemolymph from A. gemmatalis treated with opsonized and nonopsonized P. farinosus.

Discussion

The striking feature of the clearance experiments was the dramatic disappearance from hemolymph of fungi with exposed galactose residues (*Paecilomyces farinosus*) regardless of treatment. For fungi opsonized with immune hemolymph, removal was essentially complete in 30 min while in the other treatments the process occurred over 2 hr. The blastospores opsonized with normal hemolymph (which contains small quantities of lectin of uncertain carbohydrate specificity and probably also nonspecific opsonins) were more effectively cleared than BIS treated fungi but the two lines representing clearance rates were essentially parallel. In contrast, the pathogenic fungi with no exposed galactose residues (*Nomuraea rileyi*) freely circulated in the hemolymph. Although the results were not statistically significant overall, fungi treated with BIS maintained constant levels over the course of the two hr experiment while those fungi treated with either normal or immune hemolymph increased in number over the first 30 min, decreased to minimum level at 60 min and showed an increase which presumably continued over the course of the infection. At 24 hr post injection, *N. rileyi* treated insects were heavily infected with hyphal bodies while it was rare to find circulating blastospores in *P. farinosus* treated insects. In the limited number of samples examined at 24 hr, insects treated with *N. rileyi* opsonized with immune and

normal hemolymph showed a 100 fold greater level of circulating blastospores than hemolymph from insects treated with BIS-opsonized N. rileyi.

Paecilomyces farinosus is not considered to be a pathogen of A. gemmatalis while N. rileyi is a pathogen. Although all of the insects treated with the two agents succumbed, death from P. farinosus may be due to toxin production (McCoy et al., 1989) by the large number of sequestered fungi e.g., phagocytosed but not killed. It is difficult to fulfill Koch's postulates with this fungus. In only one case could P. farinosus be isolated from an infected insect. In contrast, cadavers of N. rileyi infected insects readily sporulated and the fungus could be easily isolated. These results suggest that both specific and nonspecific opsonins may be present in the hemolymph. With P. farinosus, opsonization may be mediated by the galactose lectin while, with N. rileyi, opsonization seems to be mediated by nonspecific agglutinins. The immune opsonized P. farinosus were rapidly cleared while the nonspecific opsonization by both normal and immune hemolymph of N. rileyi may cloak the cells in a mantle of self which attracts other opsonins and/or absorbs existing lectin without inducing production of additional lectin during the course of the 2 hr experiment. Results from these and other experiments suggest that lectin induction takes about 2 hr. These data represent only trends and should only be

interpreted as such. More extensive experimentation will be necessary to establish statistical significance and similar experiments using purified lectin should also allow for testing of some of the hypotheses suggested above. Until further experiments of this nature are conducted, these results will remain difficult to interpret.

Previous experimentation of this nature has been reported on S. exigua using the purified lectin obtained from this insect to opsonize P. farinosus and N. rileyi (Heath et al., 1987). Similar to findings from the present study, opsonized and nonopsonized P. farinosus were cleared by both A. gemmatalis and S. exigua and opsonization with S. exigua lectin accelerated the process with a reduction in number of blastospores by 30 min. Although opsonized and nonopsonized N. rileyi were not cleared, the number of circulating hyphal bodies was lowered by the opsonization procedure.

How this all correlates to lectin function in the insect is, of course, unknown. Since the in vitro studies have not been conducted in A. gemmatalis, these experiments can only suggest that an opsonic function might exist. If the lectin is involved in clearance, induction may involve initiation of synthesis, release from sequestration or removal of an inhibitor which leads to enhanced lectin function. In P. farinosus-treated insects, there is no evidence for fungal replication. It could be that there

are, in addition to the galactose-binding molecules which are coating the fungi, other nonspecific opsonic molecules (which may or may not include the A. gemmatalis fucose lectin) that exist naturally in the hemolymph and also stick to both opsonized and nonopsonized fungi. There is evidence from purification studies to indicate that the galactose lectin, the fucose lectin and other hemolymph proteins adhere. The relative abundance of each component in the conglomerate is unknown as is whether each component (especially the galactose lectin) is active is this state. The presence of confusing hemagglutination patterns in normal hemolymph also implies that these functions are not discrete. As suggested by the work of Bayne and Fryer (1989) and Fryer et al. (1989), any nonspecific opsonization may prevent recognition of nonself. In this experiment the fact that both immune and nonimmune opsonized N. rileyi, after a 30 min lag period, increased in number suggested that this might be occurring and the lectin induction could be triggered by fungal replication. In essence, opsonization facilitated the infection process.

Over the course of the experiment, total hemocyte count (THC) showed some increase in all treatments except immune hemolymph opsonized N. rileyi. This is in agreement with findings by Horohov and Dunn (1982) for Manduca sexta injected with bacteria. However, since the normal THC range covers the THC range found in the experiment, these results

should be viewed skeptically. In contrast to the findings by Horohov and Dunn, Gagen and Ratcliffe (1976) found that in Galleria mellonella, the THC showed a dramatic decrease following bacterial challenge. Geng and Dunn (1989), using a different population of M. sexta, failed to find the increase in THC reported earlier but instead were able to demonstrate selective depletion of plasmatocytes. Chain and Anderson (1982) reported selective depletion of plasmatocytes in bacterial challenged G. mellonella. Differential hemocyte counts were not determined in this experiment.

In contrast to the general observations from clearance studies, the data from hemagglutination tests on the same insects are obscure. The profiles of three of the six treatments resemble the normal hemolymph profile i.e., presence of prozone with heavy agglutination that appears negative, several wells with agglutination, no sharp end point, and an occasional insect with no hemagglutinin. These treatments were: BIS "opsonized" P. farinosus; normal hemolymph opsonized P. farinosus; BIS "opsonized" N. rileyi. In only one treatment (immune hemolymph opsonized P. farinosus) was there any suggestion of a trend. In this treatment, it appeared that the opsonized fungi removed HA activity from the hemolymph for at least one hr post injection. By two hr PI, there was appearance of agglutinin in the hemolymph suggesting that synthesis or release of

agglutinin might have occurred. In one other treatment (immune opsonized N. rileyi) it seemed that fungi with no exposed galactose residues removed nonspecific opsonins from the hemolymph and when injected into insects, the nonspecific opsonins removed agglutinins (which could be measured by HA) from the hemolymph. In the treatment of N. rileyi opsonized with normal hemolymph, which presumably has less opsonin, this process was less effective than that observed with immune hemolymph.

CHAPTER 6

SUMMARY AND CONCLUSIONS

This dissertation reports the progress made in purification and characterization of hemagglutinins (lectins) from the hemolymph of larval Anticarsia gemmatalis. During the course of this investigation, it was determined that the galactose-specific lectin was a component in a more complex system which also included a fucose-specific lectin or lectin-like molecule. This is the first report of an L-fucose specific lectin in an insect and the first report of a novel sequential affinity chromatography procedure to effect purification of these lectins. Both lectins appear to be composed of subunits of about 45 kd, although, the galactose lectin migrates in a diffuse manner on SDS-PAGE suggestive of heavy glycosylation and the fucose lectin appears to be composed of isolectins. The native weight of the parent molecule has yet to be accurately determined but the molecule is believed to be an octamer. The purified galactose lectin component may undergo self aggregation into molecules of MW >669,000 which do not migrate in a 4-20% native polyacrylamide gel. Although each component can be purified or partially

purified, the true native composition which occurs in insect hemolymph is unknown. Problems encountered during purification suggest that the galactose-binding proteins and the fucose-binding proteins stick together in their native state since each component is glycosylated and at least one component (the fucose-binding component) also exhibits weak mannose binding capabilities. Other data indicate that, in addition to the apparent self aggregation by the two lectin components, there is binding to other hemolymph proteins, possibly arylphorin. The method by which this lectin system functions in the intact insect is unknown but there exists some evidence that the galactose lectin, with its high specific activity and ability to recognize μg quantities of galactose, may be inactive in its native state and/or when bound to other hemolymph proteins. The fucose lectin requires the presence of Ca^{++} for activity and the depletion of this element (e.g., by starvation due to fungal infection or cessation of feeding prior to pupation) in the insect may somehow regulate the function of the galactose lectin.

Studies using monosaccharides as inhibitors of hemagglutination by these lectins have shown that there are differences in the two component lectins. When assaying purified fractions, the hemagglutinating capability of the fucose component was completely eliminated when Ca^{++} was omitted from the buffer. The galactose lectin did not exhibit a similar stringent requirement for this divalent

cation. The other major difference between the two molecules was the monosaccharide sensitivity. The galactose lectin could detect μmolar quantities of galactose while the fucose lectin required millimolar quantities. Both lectins were assayed against human erythrocytes for ABO specificity. Neither was blood group specific.

The immune hemolymph of larval A. gemmatalis can enhance clearance of fungal hyphal bodies with exposed galactose residues (Paecilomyces farinosus), but not clearance of blastospores from a fungus lacking these residues (Nomuraea rileyi). Clearance of the nonpathogenic P. farinosus did not provide protective immunity. Although not statistically significant, opsonization of N. rileyi seemed to enhance the infectivity of these hyphal bodies. These findings were similar to those of Pendland et al., 1988, who used the purified galactose lectin from Spodoptera exigua to opsonize these same fungi.

Since high titers of the lectins of A. gemmatalis were induced by injection of hyphal bodies from N. rileyi, additional studies were undertaken to ascertain 1) if other entomopathogenic and nonentomopathogenic fungi could induce similarly high titers and 2) if other microorganisms could induce the lectin system. High titers of lectin could be induced by the entomopathogenic fungus Beauveria bassiana and the nonentomopathogenic fungi P. farinosus and Candida albicans but not by bacteria or viruses.

Other studies showed that high titers of agglutinin occurred constitutively in wandering prepupae and prepupae, but not pupae. The hemagglutinin could not be induced by heat shock.

APPENDIX A

PURIFICATION METHODS STUDIED

Affi-Gel ovalbumin chelating affinity chromatography. As mentioned in Chapter 3, a purification scheme using Affi-Gel ovalbumin (BioRad, Richmond, CA) was used initially because this resin had been successfully employed by Pendland and Boucias (1986a) to purify the galactose lectin from Spodoptera exigua. An elaborate purification protocol was required and included loading immune hemolymph onto the affinity resin at 4 C in the presence of a Tris buffer containing Ca^{++} and 0.1 M NaCl, stripping away nonspecific binding by increasing the NaCl concentration to 1 M, reequilibrating the column with buffer containing 0.3 M NaCl, heating the column to 37 C and finally eluting the adhering molecules by removing the Ca^{++} with EDTA. The column was regenerated with Tris buffer containing 1 M NaCl and EDTA. When immune hemolymph from Anticarsia gemmatalis was subjected to this purification protocol, and the presumptive lectin fraction analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), the fraction was shown to consist of multiple components (data

not shown). More detailed buffer information is presented in Pendland and Boucias, 1986a.

Galactose-agarose affinity chromatography. Galactose-agarose was purchased from Pierce Chemical Co., Rockford IL. The spacer arm on this resin is diethylene sulfone. Many protocols were used with this column. In general, an ice cold aliquot of 2.5 ml immune hemolymph was diluted with 2.5 ml buffered insect saline (BIS) and applied to the column maintained at 4 C either with the aid of a peristaltic pump or by direct application to the resin with a Pasteur pipette. The binding capacity of the resin was 8-9 mg castor bean lectin per ml gel. Various methods of enhancing binding to the column were tried and included mixing the resin-hemolymph mixture with the aid of a glass stirring bar and allowing the mixture to interact for up to 30 min. Other experiments showed that this step was unnecessary and that binding of the lectin to the resin must occur very quickly. For example, if target rabbit red blood cells (RBC) were added to hemolymph immediately after dilution in inhibiting sugar when performing an HI assay, there was no difference in inhibition between these samples and those which were allowed to interact for 1 hr before addition of the RBC. When hemolymph and gel were mixed together in a flask and refrigerated overnight, there was no difference in titer between samples allowed to interact for 30 min and those allowed to interact overnight. Thus, the binding must

take place quickly, but all the hemagglutinins in the hemolymph are not able to attach to the resin. Initially, after binding of galactose-binding proteins (GBPs) and elution of non-galactose binding molecules, the GBPs were eluted with 0.4 M galactose in BIS. Such samples, when analyzed by SDS-PAGE were very impure. To remove any nonspecifically binding impurities, the column was washed with BIS buffer containing 0.5 M NaCl prior to elution with 0.4 M galactose in BIS. When the salt eluent was assayed for hemagglutination (HA), there was a consistent lack of activity in these fractions even though considerable protein (as monitored by absorbance at 280 nm) eluted. After reequilibrating the column with BIS, the GBPs were eluted with 0.4 M galactose in BIS. Initially, it was thought that the concentration of galactose might be too low but elution with 0.8 M galactose in BIS failed to improve the purification. A representative from Pierce Chemical Co. suggested that galactose-containing disaccharides such as lactose were up to 10 X more effective in displacing GBPs from the column but this was not found to improve the purification. When hemagglutination inhibition (HI) assays (using HI method I) were performed on the partially pure GBPs, L-fucose was consistently found to be a more effective inhibitor and the next purification protocols involved use of a fucose-agarose column.

Fucose-agarose affinity chromatography. Fucose-agarose was obtained from Sigma Chemical Co. The spacer arm on this resin was also diethylene sulfone and the 1 ml of the resin could bind 15-20 mg of L-fucose lectin from Tetragonobolus purpurea. The basic protocol used with the galactose-agarose column was employed. Briefly the ice cold immune hemolymph was mixed with an equal volume of BIS (2.5 ml of each) and the diluted hemolymph delivered to the refrigerated resin with the aid of a peristaltic pump, direct addition with a Pasteur pipette or by mixing the resin and diluted hemolymph with a glass stirring rod. There was more effective binding of A. gemmatalis lectin to this resin as indicated by decreased titer in the first eluent, but, as with the GBPs, the fucose-binding proteins (FBPs) must bind very quickly and none of the loading methods proved to be superior. The resin failed to remove all HA activity from the hemolymph. Molecules binding nonspecifically to the column were eluted with a BIS buffer containing 0.5 M NaCl. After reequilibrating the column with BIS, the FBPs were stripped with a BIS buffer containing 0.4 M fucose. Typical results obtained when fucose agarose samples were analyzed by SDS-PAGE, showed at least three components.

Mannose-agarose affinity chromatography. Results from a battery of HI assays using HI method I showed that there was weak inhibition by mannose. Since a mannose-inhibitable

lectin had not been reported from insects, a novel procedure involving mannose-agarose as resin for the initial purification step was used. For mannose-agarose affinity purification, an aliquot of 2.5 ml immune hemolymph was diluted with 2.5 ml BIS and applied to a mannose-agarose resin (Sigma Chemical Co.) with a binding capacity for 50-70 mg Concanavalin A per ml resin. After elution of nonspecifically binding molecules, and reequilibration with BIS, the mannose binding proteins (MBPs) were eluted with 0.4 M mannose in BIS. This method was also employed as part of a three step purification scheme where the first eluent from the mannose column was put through a galactose column for recovery of GBPs as described above. The first eluent from the galactose column was then applied to a fucose column and for recovery of FBPs as described above.

High Performance Liquid Chromatography (HPLC). An HPLC method using a protocol with trifluoroacetic acid (TFA) and acetonitrile was attempted. The acetonitrile apparently bound so strongly to the lectin that, even after the evaporation of acetonitrile, there was residual acetonitrile bound to the lectin. Hemagglutinin activity could only be restored after extensive dialysis.

Gel permeation with Bio-Gel 1.5A. Once partial purification of the lectins had been carried out by affinity chromatography, it became desirable to find a gel permeation method to effect further purification. Because of its broad

range (10 -1,500 kd), Bio-Gel 1.5A (BioRad, Richmond, CA) was chosen for study. Samples were prepared from either normal or induced hemolymph. Aliquots of 2.5 ml were diluted 1:2 in BIS, applied to a fucose-agarose column and eluted with 0.4 M fucose-BIS. Two column runs (5 ml total hemolymph) were pooled, dialyzed extensively against 1/10 BIS and 1/50 BIS and lyophilized. Each sample was reconstituted in 300 μl deionized water and 200 μl was applied to the column after dilution in an equal volume of test buffer containing 5% glycerol. The column was preequilibrated with the test buffer prior to application of sample. The various test buffer systems used with the column included: 0.2 M galactose-BIS; BIS without Ca^{++}; BIS; 0.15 M NaCl + EDTA. In addition, normal hemolymph was analyzed using a BIS buffer and an Affi-Gel ovalbumin purified sample was put through the column in the presence of 0.5 M NaCl + EDTA. Equivalents of reconstituted samples were applied to a refrigerated (4 C) jacketed column packed with BioGel 1.5A. The resin bed was 1.5 cm in diameter x 54.5 cm in length. The V_e/V_o ratios were determined from standards using a 0.5 M NaCl-EDTA buffer system. The values obtained for the standard were as follows: thyroglobulin (660 kd) 1.64; β-amylase (200 kd) 2.14; alcohol dehydrogenase (150 kd) 2.16; bovine serum albumin (66 kd) 2.2; carbonic anhydrase (29 kd) 2.43 and apoferritin subunits (18.5 kd) 2.57. Fractions of 2 ml were collected

and pooled based on HA activity and/or absorbance at 280 nm. Samples were dialyzed extensively against 1/10 BIS and 1/50 BIS and lyophilized before final analysis for protein, HA and HI (using HI method I). The samples were pooled arbitrarily and the V_e/V_o of each fraction determined from the V_e/V_o of the first and last pooled 2 ml sample. Since the conditions of the run were kept uniform except for the buffer, the V_e/V_o , were used as a basis for comparison of the fractions, each being designated by the elution position on the column. It was anticipated that pure fractions would be obtained and by comparing these values with those obtained from the MW standards, a native molecular weight determined for these molecules. As shown, neither of these goals was achieved. Figure A-1 illustrates the position on the column in which the HA activity eluted from the gel permeation column under the various conditions. The relative activity is not accurately depicted due to difficulty in portraying serial twofold dilutions. Results shown in Figure A-2 through A-6 depict protein elution profiles from the samples. Table A-1, however, illustrates in an arbitrary manner, the amount of activity recovered from each column and the inhibition by fucose and galactose using HI method I. All conditions of the runs were kept uniform, to allow for comparison of relative activity. The most efficient procedure incorporated 0.2 M galactose in the

buffer and the procedure using 0.15 M NaCl-EDTA was also quite efficient.

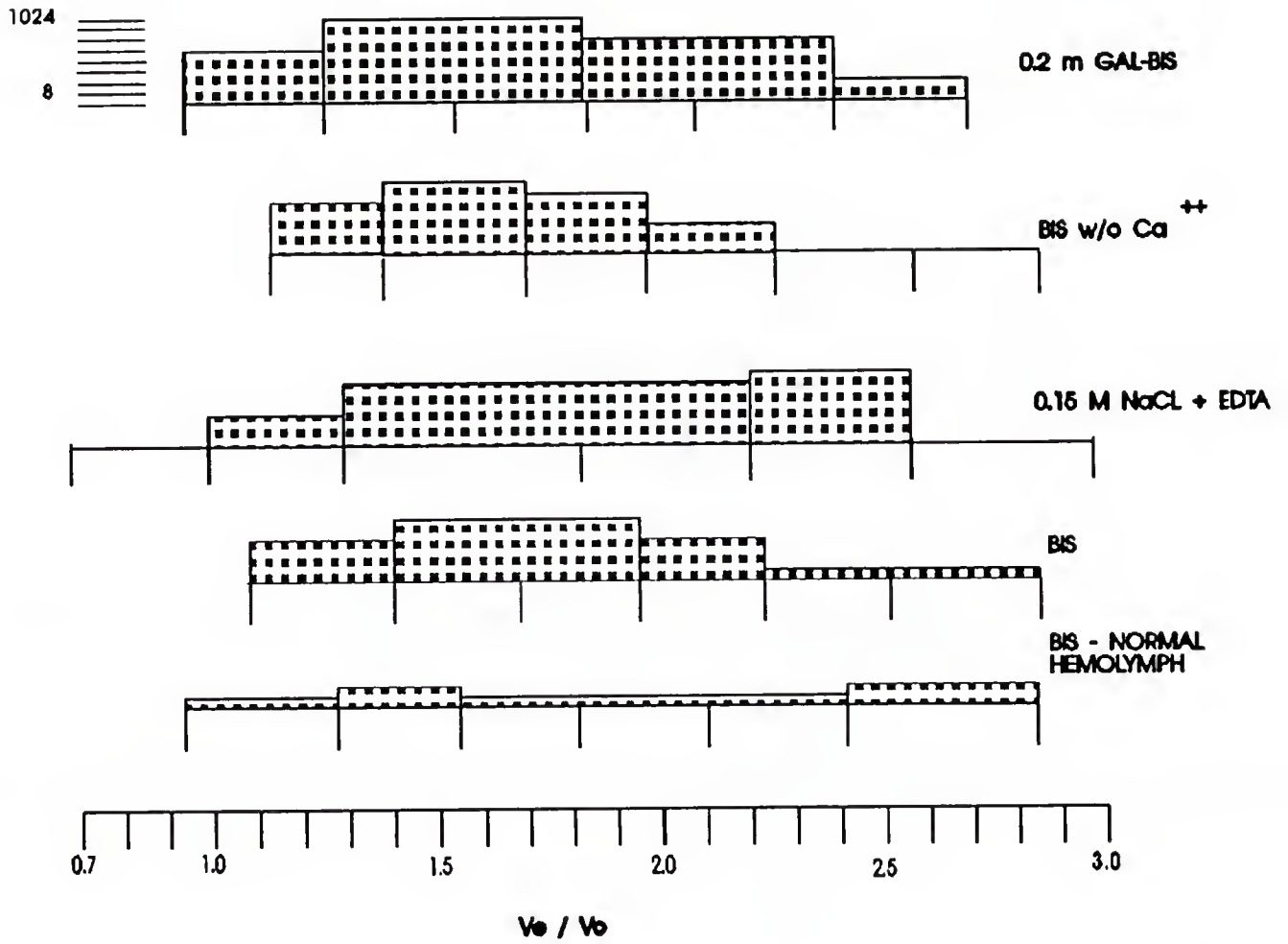


Figure A-1. Elution patterns of hemagglutinin activity from BioGel 1.5A under different buffer conditions and when analyzing normal hemolymph.

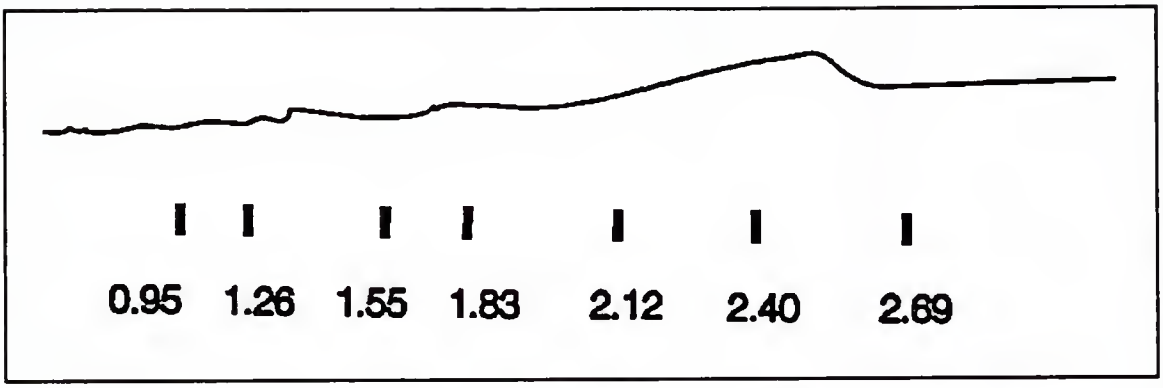


Figure A-2. Protein elution profile from BioGel 1.5A with 0.2M Gal-BIS as buffer.

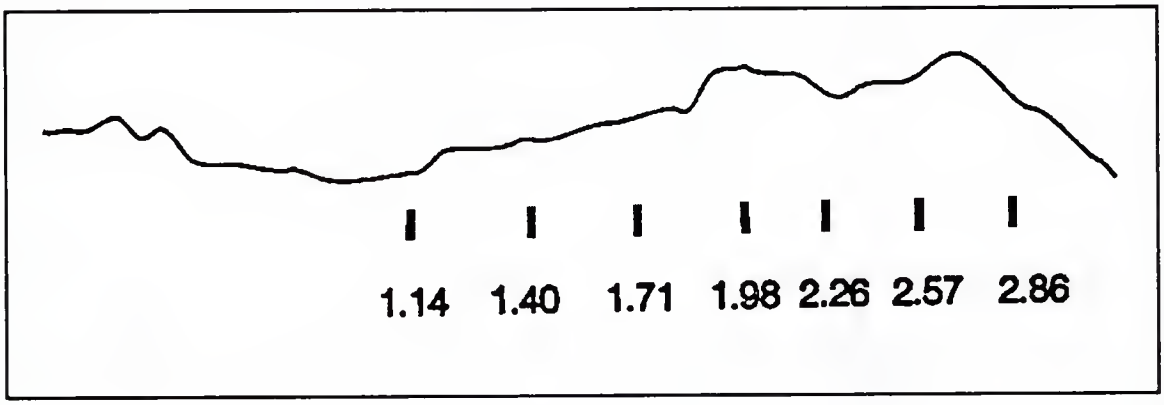


Figure A-3. Protein elution profile from BioGel 1.5A with BIS without Ca^{++} as buffer.

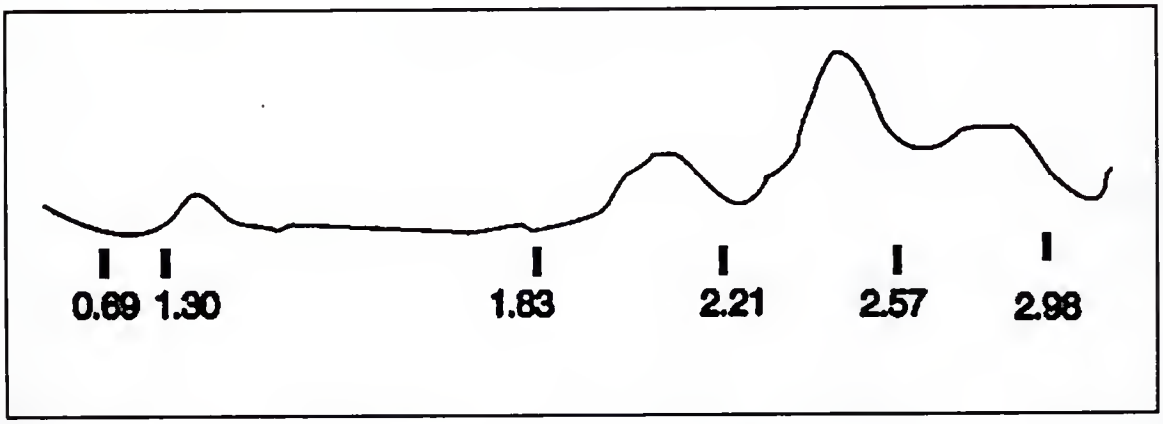


Figure A-4. Protein elution profile from BioGel 1.5A with Tris Buffer with 0.15M NaCl + EDTA as buffer.

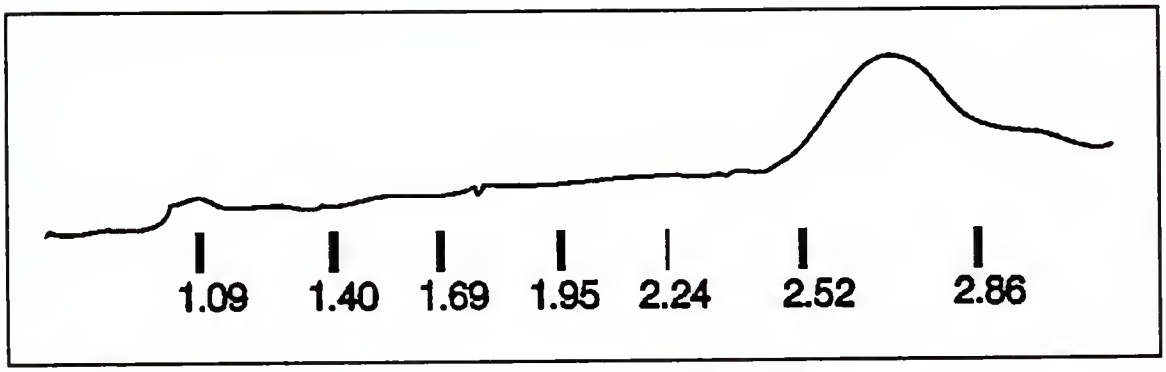


Figure A-5. Protein elution profile from BioGel 1.5A with BIS as buffer.

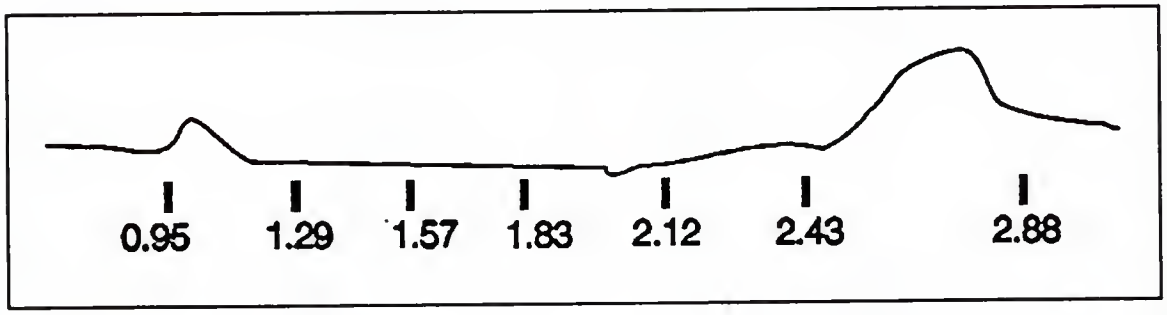


Figure A-6. Protein elution profile from BioGel 1.5A using normal hemolymph and BIS as buffer.

Table A-1. Activity recovered from BioGel 1.5A under various buffer conditions and % of activity inhibited by galactose and fucose.

Buffer	Units recovered	% inh. by Gal	% inh. by Fuc
BIS (Normal HL)	928	76	100
BIS no Ca^{++}	11136	83	100
0.15M NaCl EDTA	17344	17	100
0.2 M Gal BIS	32672	7	100
BIS	7904	31	100

APPENDIX B

MARTHA'S COOKBOOK

Insect maintenance. Colonies of A. gemmatalis were maintained in culture at the USDA Insectary in Gainesville, Fl, and the insects were collected as eggs. They were provided with artificial diet (Greene et al., 1976) in paper cups and housed in incubators at 26 C under photoperiod of 14 hr light and 10 hr dark.

Insect diet with inhibitors. A modification of the method of Greene et al. (1976) was used. For diet with inhibitors, dissolve 46 g gelacerin (HWG) and 125 g torula yeast in 3 l deionized water (preferably sterile deionized water). A wire whisk is useful for efficient mixing of the ingredients. Heat to 75 C. Do not allow the temperature to go higher. Cook at 75 C for 10-15 min with occasional stirring. While the mixture is cooking, weigh into a container the following ingredients of Mix A: 250 g pinto beans (meal); 200 g wheat germ; 100 g soybean protein; and 75 g casein. Mix the dry ingredients. In another small cup assemble the ingredients of Mix B: 12 g ascorbic acid; 20 g vitamin mix; 250 mg (1 capsule) tetracycline; 10 g methyl-p-hydroxybenzoate (methyl paraben); and 6 g sorbic acid.

Measure 15 ml 40% formalin. After the HWG and torula yeast have cooked, pour this mixture into the blender and add about half of Mix A. Put lid on blender and blend on low speed for a minute or so. Then, remove the lid and add the rest of Mix A. Remove diet from the blender rim and lid and add back to diet mixture. Turn off the blender and check the temperature. When the mixture is 67-70 C (no higher), put lid back on blender, turn on at low speed, remove lid, add formalin and let mix well. Add Mix B; clean rim and lid and put lid back on. Blend on high for 30 sec, turn off and let settle. Repeat this step several times. Pour diet into crisper and let cool with crisper lid off or slightly ajar.

Insect diet without inhibitors. Prepare as described above but make half the amount, add no formalin and add no sorbic acid.

Fungal culture maintenance. Strains of Nomuraea rileyi and other fungal cultures are stored at -70 C in the Insect Pathology Laboratory and are maintained on Sabouraud maltose yeast agar (SMY) or Sabouraud dextrose yeast broth (SDY). The strain used in this study was the FL-78 strain which was originally isolated from field collected A. gemmatalis larvae (Boucias et al., 1982).

Sabouraud maltose yeast agar. Per liter, mix 65 g Sabouraud maltose agar (Difco Laboratories) with 20 g (2%) yeast extract. Add the ingredients to slightly less than 1 l deionized water and mix with a stirring bar. Determine

the pH of the mixture at room temperature and, if necessary, adjust to pH 6.0. Adjust the volume to 1 l. With stirring, heat to dissolve the agar. Autoclave for 20 min at 15 lb pressure. Dispense into sterile Petri plates.

Sabouraud dextrose yeast broth. Per liter, mix 30 g Sabouraud dextrose broth (Difco Laboratories) with 20 g (2%) yeast extract. Add the ingredients to slightly less than 1 l deionized water and mix with a stirring bar to dissolve. Determine the pH of the mixture and, if necessary, adjust to pH 6.0. Adjust the volume to 1 l. Dispense aliquots of approximately 50 ml into 125 ml Erlenmeyer flasks and autoclave for 20 min at 15 lb pressure.

Lectin induction. For injection, fungal cells were harvested as hyphal bodies (HB) by flooding the Petri plate with sterile water. Using aseptic technique, the fungal cells were washed several times in water and suspended in sterile 0.85% NaCl. After an additional centrifugation, the cells were suspended in sterile saline with a vortex mixer. The cells were counted with a hemacytometer and diluted to the desired concentration. For preparation of high titer serum, late sixth instar A. gemmatalis were inoculated with 30,000 washed HB in sterile saline. Injections of 5-10 µl were made into a proleg with an ISCO injector (Instrumentation Specialties Co., Lincoln, NE) equipped with a tuberculin syringe fitted to a 30 gauge needle (Thomas Scientific, Swedesboro, NJ). After 24 hr, the insects were

bled by puncturing a proleg. The hemolymph was collected on a sheet of parafilm and placed on an ice bath. Hemolymph was pooled in a prechilled microcentrifuge tube containing a few crystals of phenylthiourea (PTU) and centrifuged at 10,000 x g for 5 min. The cell free hemolymph was pooled and stored at -70 C until processed.

Erythrocytes. Rabbit red blood cells (RBC) were obtained locally or from Hazelton Research Products (Denver, PA). Human RBC were obtained as outdated material from the Blood Bank at the J. H. Miller Health Center in Gainesville, FL. Blood cells were washed several times in phosphate buffered saline, pH 7.2 (PBS) and usually trypsinized prior to use according to the method of Novak et al. (1970) by treating the cells with 10 ml of a 1 mg/ml solution of TPCK digest of trypsin (Sigma Chemical Co., St. Louis, MO) in PBS, for 60 min at 37 C. After trypsinization, the cells were washed several times with PBS. The trypsin treatment caused the cells to clump and lyse easily, so they were carefully but thoroughly resuspended after centrifugation. For use, the cells were counted with a hemacytometer or the volume of packed cells determined visually. Cell suspensions of 1%, 2% or 4% were used for various experiments.

Hemagglutination Assay (HA). For assay, serial twofold dilutions of hemolymph, lectin or other test material were made in V-bottom microtiter plates using either PBS or

buffered insect saline (BIS) as diluent. Frequently, it was desirable to conserve hemolymph or purified lectin and 10 µl of hemolymph were added to 70 µl diluent giving a starting dilution of 1:8. Then, 50 µl were transferred to a well containing 50 µl diluent and, subsequently, serial twofold dilutions made. In the first well, 30 µl of target RBC were added. An equal volume (usually 50 µl) of erythrocytes was added to the following wells and after an incubation of 1 hr at room temperature, the plates were read. The plates were refrigerated and reread after several hours or overnight. A positive reaction appeared as a diffuse mat in the bottom of the well and a negative reaction appeared as a compact red dot. Positive and negative controls were included in each group of assays. The titer was expressed as the reciprocal of the highest dilution giving complete HA.

Hemagglutination Inhibition (HI) Assay I. Sugars were usually obtained from Sigma Chemical Co. (St. Louis, MO) and were of reagent grade. Solutions of test sugar were prepared as 200 mM solutions in either PBS or BIS. Using V well microtiter plates, serial twofold dilutions of hemolymph or lectin were prepared and after an incubation of 1 hr at room temperature, an equal volume of a 1-2% solution of test RBC were added. After an additional incubation at room temperature for 1 hr, the plates were read. Plates were reread after several hours or an overnight incubation at 4 C. Positive and negative controls were included with

each group of assays. The titer was recorded as the reciprocal of the highest dilution (lowest concentration of hemolymph or lectin) giving complete inhibition.

Hemagglutination Inhibition (HI) Assay II. For this assay, the hemagglutination titer of the hemolymph, lectin or test substance was determined and considered one unit e.g., with a titer of 1024, a 1:1024 dilution yields one unit. For HI, four units were used e.g., a 1:256 dilution. Test monosaccharides were usually prepared at a concentration of 800 mM in BIS or occasionally in PBS. Other sugars such as disaccharides, relatively insoluble sugars or expensive sugar derivatives were prepared at lower concentrations. To the first well of the microtiter plate were added 50 µl of test sugar. In the second well 50 µl of test sugar solution were added to the 50 µl diluent (usually BIS) and serial twofold dilutions made. To each well were added 50 µl of solution containing 4 units hemagglutinin. After an incubation of 1 hr at room temperature, a solution of 4% RBC was added. The HI assay was read after the 1 hr incubation and after refrigeration for several hours or overnight. Positive and negative controls were included with each assay and consisted of hemagglutinin + RBC, inhibiting sugar + RBC or diluent + RBC. In calculating the minimum inhibitory concentration (MIC), the dilution of stock sugar solution, allowing for dilution by hemagglutinin or diluent was recorded as the MIC. Since four units of

added lectin contained adequate agglutinin, no dilution factor was considered. This was also true for the indicator system - the test RBC.

Phosphate buffered saline (PBS) pH 7.2.

Solution A - 0.2 M Na_2HPO_4-$7H_2O$ (53.65 g/l)

Solution B - 0.2 M NaH_2PO_4-H_2O (27.6 g/l)

Mix 36 ml solution A, 14 ml solution B and 0.438 g NaCl. Dilute to 100 ml with deionized water.

Buffered Insect Saline (BIS). (Castro et al., 1987) pH 7.89 at 5 C; use Sigma formula for 0.01 M Tris at 5 C.

	1 liter
Trizma HCl	1.370 g
Trizma base	0.16 g
130 mM NaCl	7.597 g
1 mM $CaCl_2$-2 H_2O	0.147 g
5 mM KCl	0.373 g
Na azide	0.2 g

For 0.5 M NaCl buffer, omit the NaCl and add 29.22 g/l to the basic buffer. All buffers for chromatography were prepared in deionized degassed water and filtered through a 0.2 μ filter. Buffers were stored at 4 C with azide and discarded if contaminated.

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BIOGRAPHICAL SKETCH

Martha A. Heath is the daughter of Mrs. Helen Hutchinson and Mr. Ivan Hutchinson. Martha was born on January 9, 1945, in Toledo, OH. She graduated from Whitmer Senior High School in Toledo, OH, in June 1963, attended the Ohio State University and graduated from the University of Toledo in January 1967. After working briefly for the Bureau of Radiological Health, USPHS, she continued her education at the University of Maryland and received the degree M.S. with a major in microbiology (immunology) in August 1970. After receiving her M.S., she worked in private industry as an immunologist for Canalco. In 1985, after an extended hiatus, Martha continued her education at the University of Florida in pursuance of a Ph.D. in entomology (insect pathology). She is married to Robert Heath and they have two daughters, Elizabeth and Catherine. She is the daughter-in-law of Mr. and Mrs. Ralph Heath.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Drion G. Boucias, Chair
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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